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The Roles of Mineralocorticoid and $GABA_A$ Receptors in Anxiety and Fear Memory

by

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Abstract

The purpose of this dissertation was to examine the roles of brain mineralocorticosteroid (MR) and γ-aminobutyric acid ($GABA_A$) receptors in mediating unconditioned fear and fear memory. The first set of experiments explored the role of hippocampal and medial prefrontal cortex mineralocorticosteroid receptors (MRs) in anxiety and fear memory. The MR antagonist RU28318 was microinfused into the dorsal hippocampus (DH), ventral hippocampus (VH) or medial prefrontal cortex (mPFC) ten minutes prior to testing in two rodent models of unconditioned anxiety, the elevated plus-maze and shock-probe burying test. Fear memory was then assessed in the shock-probe apparatus 24 hours later by re-exposing non-drugged rats to a non-electrified probe. RU28318 infusions into the VH reduced anxiety in the elevated plus-maze while RU28318 in the DH or mPFC did not. In contrast, RU28318 infusions into the DH, VH and mPFC all reduced anxiety in the shock-probe burying test. Fear memory was not affected by infusions into any of the three brain regions. The second and third set of experiments examined the role of hippocampal $GABA_A$ receptors and $GABA_A$ receptor sub-units in mediating anxiety and fear memory. α2 $GABA_A$ receptor sub-units are thought to mediate the anxiolytic effects of benzodiazepines and α5 sub-units are thought to mediate the amnesic effects of benzodiazepines. The DH and VH both contain $GABA_A$ receptors having these sub-units. Rats were given intra-hippocampal microinfusions of either TPA023 (an α2 agonist) or TB-21007 (an α5 inverse agonist) and tested in the plus-maze and shock-probe tests. Twenty-four hours later, rats were tested for fear memory

with the non-electrified shock-probe. The α2 agonist (TPA023) reduced anxiety when it was infused into the VH but had no effect when infused into the DH. Conversely, the inverse α5 agonist (TB-21007) impaired fear memory when it was infused into the DH, but not when it was infused into the VH. Overall, these results suggest that mineralocorticoid and $GABA_A$ receptors in the ventral hippocampus mediate anxiety. In addition, these results suggest that ventral hippocampal $GABA_A$ α2 sub-units mediate anxiety and dorsal hippocampal $GABA_A$ α5 sub-units mediate fear memory.

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Chapter 1

Introduction

When an animal is anxious or fearful, a cascade of adaptive reactions occur, in which "stress" hormones are released, respiration and heart rate is increased, sweating is increased and digestive processes are suppressed. In encounters with fearful stimuli, animals will engage in defensive behaviours. These behaviours allow the animal to avoid or escape the acute, threatening stimuli (Tinsley and Fanselow 2005). Many of these defensive behaviours (e.g., fighting versus escaping fearful stimuli) are similar in humans and other animals (Blanchard et al. 2011). Remembering places in which dangerous stimuli are encountered is also an adaptive mechanism that enhances the likelihood of survival (Wilson et al. 2011). Thus, unconditioned anxiety and fear memory are closely related adaptive mechanisms by which animals avoid life threatening sources of harm.

Anxiety disorders

When anxiety or fear become extreme, long lasting, and out of proportion to any actual threat, so much so that it interferes with daily life, it is classified as an anxiety disorder (DSM IV-TR). Anxiety disorders are the most common form of psychiatric disorder. It is estimated that approximately 30% of the general population will experience an anxiety disorder during their life time (Kessler et al. 2005). There are five main anxiety disorders listed in The Diagnostic and Statistical Manual of Mental Disorders, as well as minor derivatives which I will not describe here (see DSM IV-TR).

1. *Panic Disorder* includes recurrent and unexpected panic attacks. During a panic attack, persons often experience sweating, shortness of breath,

nausea, dizziness, a feeling of losing control, fear of dying, and derealisation. Fear about having a panic attack in a public area can result in persons avoiding public places previously associated with an attack (agoraphobia).

2. *Generalized Anxiety Disorder (GAD)* is characterized by excessive anxiety and worry that is not associated with any specific danger or threat. As a result, persons may experience impaired concentration, insomnia, and increased restlessness, irritability, and muscle tension. If three of these symptoms are experienced most days for a period of six months then a diagnosis of *GAD* may be established.
3. *Obsessive Compulsive Disorder (OCD)* includes obsessions which entail thoughts, images or impulses that persistently recur in a persons' conscious experience. These obsessions increase anxiety. The disorder is also characterized by compulsions which include ritualistic behaviours or mental acts undertaken by the person to alleviate anxiety resulting from these intrusive and persistent obsessions.
4. *Post Traumatic Stress Disorder (PTSD)* is characterized by a traumatic event (e.g., war) in which a person experiences extreme horror and fear of dying. The person re-experiences the event via flashbacks which occur in response to internal or external cues that remind the person of the original traumatic event. The individual then attempts to avoid thoughts or activities which may serve as a reminder of the trauma. In addition, persons may experience difficulties with sleep and concentration along

with increased irritability, hypervigilance and exaggerated startle responses.

5. *Specific Phobias* entail excessive fears of specific objects or situations, which the person knows are unreasonable. These objects or situations may include flying in an airplane, exposure to insects, receiving an injection, seeing blood, and so forth. Persons with specific phobias will take extensive measures to avoid the fearful objects or situations (e.g., avoid travel on airplanes). When exposed to the phobic stimuli persons experience an immediate fear response.

Pharmacotherapy of anxiety disorders

Modern pharmacotherapy for anxiety disorders began with the discovery of barbiturate drugs in the mid-nineteenth century and their clinical use in the early twentieth century (Lynton 2007). Barbiturates are allosteric $GABA_A$ receptor agonists that bind to a separate site located within the $GABA_A$ receptor protein (Leeb-Lundberg et al. 1980; Uusi-Oukari and Korpi, 2010). When barbiturates bind to $GABA_A$ receptors they increase the *duration* of $GABA_A$ chloride channel openings (Study and Barker 1981), which facilitates the inhibitory effect of GABA at the $GABA_A$ receptor (Olsen et al. 1986). This general chain of causal events is thought to produce the anxiolytic effects of other positive allosteric modulators of the $GABA_A$ receptor such as benzodiazepines, alcohol, and certain neurosteroids (Rudolf 2001). With respect to barbiturates, however, it soon became apparent that rapid tolerance developed to their anxiolytic and sedative effects, which prompted the use of higher doses, which

often led to physical dependence, and in some cases, addictive behavior (Jacob et al. 1979). As the dose of barbiturates was increased to maintain their therapeutic (or intoxicating) effects, the toxic effects of barbiturates would inevitably emerge. These could include bradycardia, hypothermia, coma, and respiratory collapse. The distance between the therapeutic and toxic doses of barbiturates is narrow, and eventually led to their clinical abandonment as a treatment for human anxiety disorders. Associated with these developments was a widespread search for a safer, more effective compound for the treatment of human anxiety.

In the 1960s benzodiazepines were introduced for the treatment of anxiety disorders. Currently around fifty types of benzodiazepines are used in medical practice world-wide (Farrell and Fatovich 2007). Benzodiazepines, like barbiturates, are allosteric $GABA_A$ agonists (Gavish and Snyder 1980; Leeb-Lundberg et al. 1980). Benzodiazepines, however, bind at a different site on the $GABA_A$ receptor than do barbiturates (Uusi-Oukari and Korpi 2010). When benzodiazepines bind to this receptor site they produce an increase in the *frequency* of $GABA_A$ chloride channel openings, in contrast to barbiturates which increase the *duration* of chloride channel openings (Study and Barker 1981). This increase in the frequency of chloride channel openings also facilitates the inhibitory effects of GABA at the $GABA_A$ receptor, producing the anxiolytic, muscle relaxant and amnesic effects of benzodiazepine drugs such as diazepam. In spite of their rise to clinical popularity in the early 1980s, when benzodiazepines were among the most prescribed drugs in the world (e.g., 100,000,000 prescriptions/yr in the US alone), it gradually came to be recognized

that, as with chronic barbiturate use, chronic benzodiazepine use also produces tolerance and withdrawal, leading to physical dependence (Dell'osso and Lader 2012). Benzodiazepines are currently used to treat episodes of acute anxiety (e.g., panic disorder) but are generally avoided for the long term treatment of other anxiety disorders (Pollack et al. 2008). Benzodiazepines are also not effective at treating co-morbid disorders often associated with anxiety disorders such as depression (Dunlop and Davis 2008).

Partial agonists of the 5-HT_{1A} serotonin receptor sub-type were introduced in the 1980s for the treatment of anxiety disorders (e.g., buspirone). These partial agonists bind to $5HT_{1A}$ receptors which in turn inhibit cellular responses. $5HT_{1A}$ agonists are thought to produce anxiolysis in part by activating *pre-synaptic* $5HT_{1A}$ receptors (DeVry 1995; DeVry et al. 2004). Clinical evidence gained over the past three decades, however, indicates that these medications have limited efficacy in treating most anxiety disorders with the exception of generalized anxiety disorder (Rendleman and Walkup 2011). $5HT_{1A}$ partial agonists are also associated with several unwanted side effects including insomnia, dizziness and nausea (Huffman et al. 2008).

Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine were also introduced for the treatment of anxiety in the 1980s. SSRIs are effective at treating most anxiety disorders, as well as co-morbid disorders such as depression (Dunlop and Davis 2008). However, these drugs have several undesirable side effects including sexual dysfunction, weight gain, insomnia, hyponatremia, hypertension, sedation, nausea, somnolence, and anxiety, as well as physical

withdrawal after cessation of therapy (i.e., "discontinuation syndrome") (Pollack et al. 2008). Although both SSRIs and benzodiazepines are effective in treating human anxiety, they suffer from important limitations that can reduce and sometimes obscure their clinical benefit.

Animal models of anxiety

Animal models of anxiety attempt to satisfy three conditions, namely: 1) that the stimulus used to induce anxiety is effective for both animals and humans (e.g., painful stimuli); 2) that the anxiety reactions to these fearful stimuli are similar in humans and animals (e.g., sudden withdrawal or escape); and 3) that clinically proven anxiolytic compounds specifically reduce these fear reactions in both the animal model and the target patient population (anxious humans). These three principles are also known as homology, isomorphism and correlation, respectively (Treit 1985; Treit et al. 2010). The intention of this section is to briefly describe a selected number of animal models of anxiety and to address their advantages and disadvantages. This section is not intended to provide an exhaustive discussion of animal models of anxiety.

'Conflict' tests were introduced in the 1960s (Geller and Seifter 1960) and are currently used as animal models of anxiety. In these tests behavioural responses to 'conflicting' or opposing drives are measured. For example, in one version of the conflict test water-deprived rats are first trained to receive a *reward*, namely water, by licking a water-filled tube. Once this rewarded behavior is established, a 'conflict' is induced by pairing rewarded licking behaviour with a *punishing* stimulus, namely painful electric shock. Not surprisingly this results in

a reduction of rewarded licking behavior, which is assumed to be mediated by conflicting drives or motivational states. Anxiolytic compounds including $GABA_A$ agonists and antidepressants *increase* the number of punished responses made by these water-deprived rats (i.e., the drug is said to have an "anti-conflict" or "anti-punishment" effect) (e.g., Kapus et al. 2008; Mathiasen and Mirza 2008; Vigliecca 2007). On the other hand, non-anxiolytic compounds such as antipsychotics and analgesics generally do not affect punished responding in these tests (e.g., Millan et al. 1999; Mathiasen and Mirza 2005). In combination, these results show that the anti-conflict test is selectively sensitive to anxiolytic drugs, which supports its "predictive" validity (Treit 1985).

Nevertheless, conflict tests are associated with a number of limitations. For example, drugs that affect rats' motivation to eat or drink may increase or decrease punished responding in these tests through changes in appetite rather than anxiety (Treit 1985; Treit et al. 1987; Hanson and Nemeroff 2009; Koob and Zimmer 2012). Another problematic drug effect is that produced by analgesics. Here, a reduction in pain sensitivity or pain perception can appear to be an anxiolytic effect when it is not. Although additional tests can be conducted to try to eliminate these alternative interpretations of what appear to be anti-conflict drug effects, the process is often long and cumbersome, and in some cases, almost impossible. For example, all conflict tests require that animals learn and remember a response (e.g., bar-pressing) through prior training. Therefore, compounds that have effects on learning and/or memory (e.g., the amnesic effects of benzodiazepines) may appear to facilitate or alter the punished responses

through a reduction in anxiety when in fact the "anti-conflict" effect is due an anti-memory effect (Millan and Brocco 2003). Therefore, learned responses reinforced with food or water or punished with electric shock actually represent a precarious platform for the interpretation of anxiolytic drug effects.

In light of these problems, several ethologically-inspired animal tests have been developed to model anxiety. These tests measure animals' naturally occurring, unconditioned fear responses to ethologically relevant sources of fear. Unlike conflict tests, ethologically-inspired models do not depend on previous training and are not confounded by motivational factors (e.g., the motivation to eat or drink; Treit 1985; Koob and Zimmer 2012). Two widely used and well-validated ethologically-inspired models of unconditioned anxiety are the elevated plus-maze and shock-probe burying test. Drugs that effectively reduce anxiety in humans (e.g., benzodiazepines) reliably inhibit rats' fear behaviours in these tests (Pellow et al. 1985; Treit et al. 1981).

The elevated plus-maze (Pellow et al. 1985) consists of four elevated platforms (arms) of which two are open and two are enclosed by walls. Rats normally spend the majority of time in the closed arms, while avoiding the open arms. The proportion/percentage of time spent in the open arms and the proportion/percentage of open-arm entries are used as measures of anxiety. Increases in these measures of open arm-activity indicate anxiolysis. This five-minute test can be used to screen dozens of candidate compounds for the treatment of anxiety in a much shorter time frame than offered by traditional conflict tests, and the results are not confounded by drug effects on pain-

sensitivity, food-motivation, or learning and memory. The total number of arm entries of any kind and the number of closed-arm entries are common measures of general activity in the plus-maze.

The shock-probe burying test (Pinel and Treit 1978) consists of a Plexiglas chamber with bedding material (wood chips) spread evenly over the chamber floor. A Plexiglas shock-probe is helically wrapped with two copper wires and inserted through a hole in one of the walls of the chamber. The probe is then electrified. Rats are placed in the Plexiglas chamber, facing away from the electrified probe. During the fifteen minute test, rats typically receive one or two contact-induced shocks from the electrified probe, which normally elicits "burying behavior" in which rats spray bedding material toward or over the probe, with rapid, alternating movements of the forepaws, while avoiding further contact with the shock-probe. A reduction in the duration of probe-burying, in the absence of a decrease in general activity or shock-sensitivity is used as the primary index of anxiety reduction. A reduction in simple passive avoidance of the probe can also be used as a measure of anxiety reduction in this test. Memory of the initial shock-probe experience can be assessed after a 24-hour delay, where rats are re-tested in the same chamber in which the probe-shock occurred, except that the probe is not electrified. The amount of time rats spend in the half of the chamber farthest away from the shock-probe is taken as a measure of fear memory.

The hippocampus, anxiety and memory

The patient HM provided important evidence for the role of the hippocampus in mediating declarative or conscious memory in humans. HM was

a patient who suffered intractable epileptic seizures. In order to ameliorate this seizure activity, sections of HMs temporal lobes were removed, including large portions of his hippocampus. Although HMs seizure activity was largely eliminated after surgery, he suffered severe anterograde amnesia as a result. Specifically, HM was unable to form new declarative memories of events that he encountered daily despite repeated exposure to these events (Milner 1972). For example, he was unable to remember experiences with hospital staff he encountered daily over many years, insisting that these were novel. Despite these memory impairments, HM was able to acquire procedural tasks (e.g., the mirror drawing task) after many practice sessions without any explicit recall of encountering these tasks previously (Milner 1965). These studies with the patient HM strongly suggest that the hippocampus plays an important role in mediating declarative or conscious memory.

Animal studies have demonstrated that the hippocampus also mediates other specialized types of memory, such as spatial memory. The Morris water maze is a widely used measure of spatial memory. The maze consists of an open pool filled with murky water. Rats must locate and then subsequently remember the location of a submerged platform via landmark or spatial cues in the environment (Morris 1984). Rats that have lesions to the hippocampus are impaired in this task. They are unable to find the location of the submerged platform during a retention test, in contrast to non-lesioned controls (e.g., Morris et al. 1990). These findings and other subsequent studies have reinforced the idea that the hippocampus mediates spatial memory (Burgess et al. 2008).

In addition to its role in mediating memory, accumulating evidence suggests that the hippocampus also mediates emotions such as anxiety. James Papez (1937) an anatomist, was first to argue that a specific neural circuit explained how emotions are mediated by the brain. In this model the hippocampus, in connection with other brain areas (i.e., the hypothalamus and cingulate cortex), mediated emotion. MacLean (1949), a psychiatrist, later added other brain structures to this 'Papez circuit' including the septum, amygdala and prefrontal cortex. MacLean further proposed that the hippocampus translated "visceral" non-verbal information received from limbic areas into conscious emotional experiences (MacLean 1949). Thirty-three years later, Jeffrey Gray (Gray, 1982) elaborated the role of the hippocampus in emotion. Specifically, Gray suggested that the hippocampus and septum work in concert to mediate anxiety. Treit and his students provided the first, direct empirical support for Gray's theory by showing that temporarily inactivating or lesioning the septum or hippocampus reduced anxiety in well-validated animal models of anxiety (e.g., Treit and Pesold 1990; Menard and Treit 1996; Pesold and Treit 1996; Degroot and Treit 2003; McEown and Treit 2009).

More recent studies have suggested that different regions of the hippocampus separately mediate anxiety and memory (e.g., Kjelstrup et al. 2002; Bannerman et al. 2004; Fanselow and Dong 2010). Simply put, the dorsal hippocampus is thought to mediate memory and the ventral hippocampus is thought to mediate anxiety (Bannerman et al. 2004). Lesioning or temporarily inactivating the dorsal hippocampus impairs spatial memory (e.g., Eijkenboom

and Van Der Staay 1999; McHugh et al. 2008), whereas lesioning or temporarily inactivating the ventral hippocampus impairs unconditioned fear behaviour, or anxiety (McEown and Treit 2009; 2010; Pentowski et al. 2006). This neat, functional dissociation between the dorsal and ventral hippocampus, however, is not always supported by other research (Engin and Treit 2007; Fanselow and Dong 2010). Some research even suggests that the apparent dichotomy in function between the dorsal and ventral hippocampus is actually a continuum of functional variation along its dorsal—ventral axis (e.g., Yartsev 2010).

The hippocampus: general anatomy and neurochemistry

The hippocampal formation consists of six sub-regions. These sub-regions include the dentate gyrus (DG), hippocampus proper (CA1, CA2 and CA3), subiculum-presubiculum, parasubiculum and entorhinal cortex (EC). This 'c' shaped brain structure emerges dorsally from the septal forebrain and continues ventral-laterally along the medial temporal lobe. The hippocampus proper (CA1, CA2, CA3) and dentate gyrus are located at the septal pole of the hippocampal formation. The remaining sub-regions (the subiculum-presubiculum, parasubiculum and entorhinal cortex) are located near the temporal lobes. Figure 1-1 displays a schematic of the hippocampus.

The hippocampus is largely a unidirectional circuit. Inputs into the circuit originate from the entorhinal cortex via the perforant pathway and travel to the dentate gyrus. Neuronal impulses then flow from the dentate gyrus via the Mossy fiber pathway to CA3. Conduction then continues from CA3 via the Schaffer Collateral pathway to CA1. The output from the circuit ends in the entorhinal

cortex through the subiculum thereby completing the circuit. Transecting the hippocampus through the horizontal longitudinal axis divides the structure into dorsal (upper) and ventral (lower) regions relative to the horizontal axis (Moser and Moser 1998). Approximately 90% of neurons within the hippocampus are excitatory (i.e., Glutamatergic) and 10% are inhibitory, consisting of GABAergic interneurons (Freund and Buzsaki 1996). Hippocampal neurons contain receptors for GABA, corticosteroids, glutamate, acetylcholine, norepinephrine, serotonin and dopamine (Kohler et al. 1991; Stark et al. 1975; Vizi and Kiss 1998).

Stress responses, anxiety and the hippocampus

When animals are confronted with fearful stimuli two stress response pathways are activated. The first stress response system is the sympathetic – adrenal medulla system. When the sympathetic nervous system is activated acetylcholine (ACh) is released from presynaptic sympathetic buttons, which then activate receptors of the adrenal medulla, resulting in the release of epinephrine. ACh released from these presynaptic buttons also bind to various sympathetic ganglia in the PNS which then release norepinephrine. These neurotransmitters induce physiological stress reactions which include increased sweating, increased breathing, and increased heart rate and decreased digestive responses.

The second stress response system is the hypothalamic-pituitary-adrenal system (HPA). Corticotropin releasing hormone (CRH) is released from the hypothalamus where it enters the hypothalamo-portal pituitary circulatory system. CRH then binds to receptors in the anterior pituitary gland which in turn releases adrenocorticotropic hormone (ACTH) into the blood stream. ACTH then binds to

adrenal cortex receptors which facilitates the release of cortisol (corticosterone or CORT in rats) into the blood stream. CORT then passes the blood brain barrier where it binds to mineralocorticosteroid (MR) and glucocorticosteroid (GR) receptors located in the brain.

Both MRs and GRs are located in areas of the brain known to mediate anxiety or fear, such as the amygdala and hippocampus (Joels et al. 2008; Prager et al. 2010; Sarabdjitsingh et al. 2009). The hippocampus is thought to act as an inhibitory mechanism in response to stress hormones such as CORT by indirectly reducing the release of CRH via the hypothalamus in response to CORT binding within the hippocampus (Feldman et al. 1995; Jacobson and Sapolsky 1991). When CORT binds to receptors in the hippocampus less CRH is released. The amygdala, on the other hand, performs the opposite function. It indirectly *potentiates* the release CRH from the hypothalamus in response to high levels of CORT binding to MRs and GRs in the amygdala (Feldman et al. 1995). This intricate system ensures the regulation of CORT levels in the body by inhibiting or potentiating the release of CORT depending on the needs of the animal (Herman et al. 2005).

However, if stress is prolonged or chronic this regulatory system breaks down. When animals are exposed to chronic stressors their adrenal gland becomes overactive and basal CORT levels rise (Engler et al. 2005). The hippocampus then becomes more susceptible to cell death (Conrad 2008) thereby impairing inhibitory control over further CRH and subsequent CORT release. Decreased neurogenesis in the hippocampus is also documented in response to increased

CORT levels (Cameron and Gould 1994). Some evidence suggests that this impaired neurogenesis is associated with increased anxiety and depression (David et al. 2010). Neural mechanisms in the hippocampus clearly play a central role in mediating stress responses via the HPA axis. However, the precise role that these underlying systems in the hippocampus play in mediating anxiety and fear memory are not well understood.

Thesis rationale

The purpose of this thesis is to explore, in three sets of experiments, two receptor systems found in the hippocampus that are implicated in anxiety and fear memory. The first set of experiments will explore the role of MRs in mediating anxiety and fear memory by infusing an MR antagonist (RU28318) into the dorsal or ventral hippocampus. The second set of experiments will explore the role of $GABA_A$ receptors in the dorsal and ventral hippocampus on anxiety and fear memory. This will be accomplished first by infusing a non-selective $GABA_A$ agonist (diazepam) and non-selective $GABA_A$ inverse agonist (FG-7142) into the dorsal or ventral hippocampus. Then, in the third set of experiments, the role of specific hippocampal $GABA_A$ receptor sub-units in mediating anxiety and fear memory will be explored by infusing two $GABA_A$ sub-unit specific compounds (TPA023 or TB-21007) into in the dorsal or ventral hippocampus.

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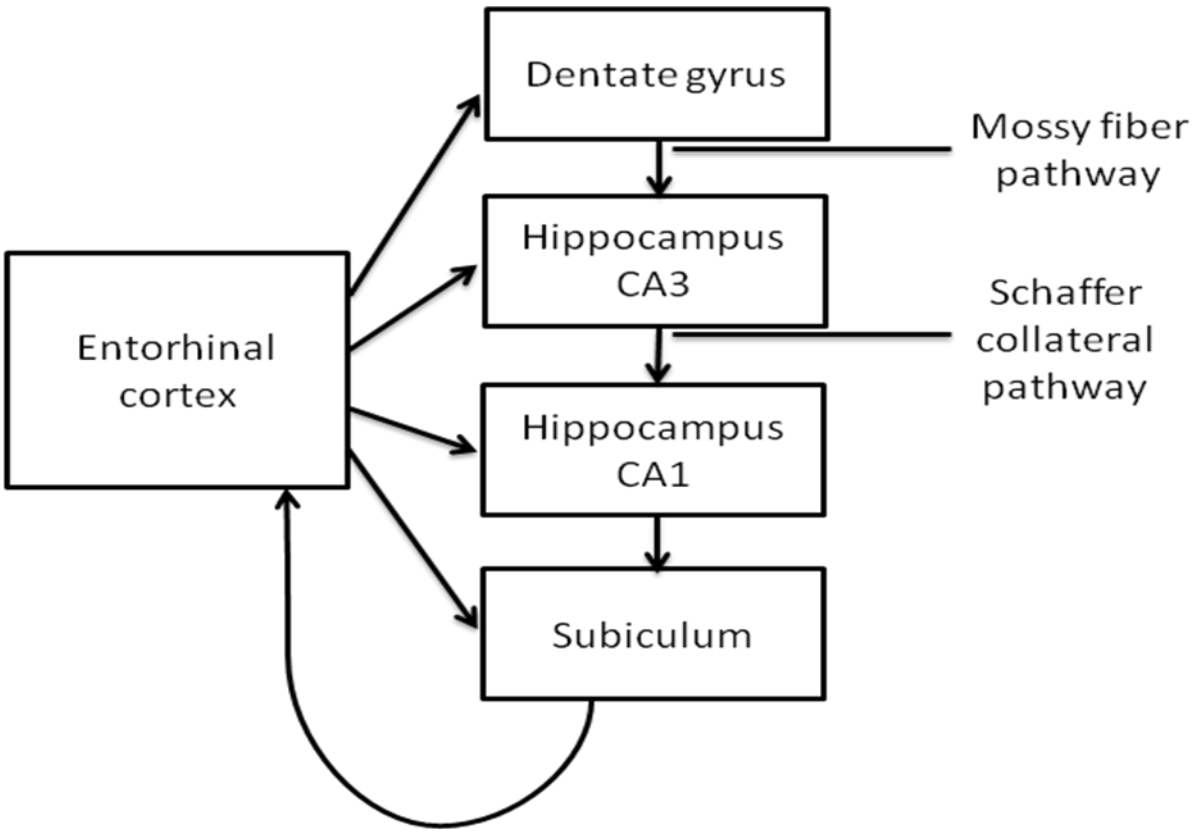
Figure 1-1: Schematic of the hippocampus adapted from Kandel et al. 2000.


Dentate gyrus
Mossy fiber pathway
Hippocampus CA3
Schaffer collateral pathway
Entorhinal cortex
Hippocampus CA1
Subiculum

Chapter 2

Mineralocorticoid Receptors in the Medial Prefrontal Cortex and Hippocampus Mediate Rats' Unconditioned Fear Behaviour

A version of this chapter is published in *Hormones and Behavior*, 60, 581-588.

1. Introduction

Differences have been found in the regional architecture, connectivity, neurophysiology, and neurochemistry of the dorsal and ventral hippocampus (e.g., Dong et al., 2009). These differences suggest functional specializations might also exist in the two areas (Dong et al., 2009; Fanselow and Dong, 2010; Royer et al., 2010; Sotiriou et al., 2005; Engin and Treit, 2007). One proposal is that the ventral hippocampus mediates fear behaviour, while the dorsal hippocampus mediates memory (e.g., Bannerman et al., 2004; Pentkowski et al., 2006). McEown and Treit (2009; 2010) provided evidence for this view using the defensive burying test (Pinel and Treit, 1978), an animal model of rat "anxiety" (Treit et., 1981; 2010). They found that temporary lesions of the ventral hippocampus impaired rats' immediate, unconditioned fear behaviour directed to an electrified probe, whereas dorsal hippocampal lesions did not. Dorsal hippocampal lesions, however, interfered with rats' memory of the shock-probe during a 'retention test' given 24hr later, whereas ventral lesions did not. These double dissociations occurred whether neuronal inactivation of the hippocampus was induced by sodium channel blockade (McEown and Treit, 2009) or by post-synaptic $GABA_A$ receptor- mediated inhibition (McEown and Treit, 2010).

The hippocampus is also a key mediator of the hypothalamic-pituitary-adrenal cortex (HPA) 'stress' pathway. Corticosteroid hormones (cortisol in humans) are released from the adrenal cortex, cross the blood-brain-barrier, and ultimately bind to glucocorticoid (GR) and mineralocorticoid (MR) receptors in the hippocampus. Binding of hippocampal GRs and MRs inhibits further

activation of the HPA, thus completing a negative feedback loop (Herman et al., 2003). While GRs are widely expressed throughout the brain, MR receptors are more concentrated in 'limbic' areas, such as the hippocampus, medial prefrontal cortex, amygdala, and septum (Van Eekelen et al., 1988). These particular limbic structures have well-documented roles in the expression of anxiety in animal models (e.g., Menard and Treit, 2000; LeDoux, 2000; Shah et al., 2004a,b; Shah and Treit, 2004; Degroot and Treit, 2004).

Nevertheless, potentiation or inhibition of MR receptors in these regions has by and large had inconsistent effects. For example, combined intracerebroventricular (ICV) infusions of MR and GR antagonists produced an increase in anxiety, as measured by the defensive burying test and the fear potentiated startle test, but each antagonist by itself had no effect (Korte et al., 1996). In direct contrast, Bitran et al. (1998) showed that microinfusion of an MR antagonist into the dorsal hippocampus produced significant anxiolytic effects in the defensive burying test, as well as the elevated plus-maze test, and the open field test, while combined infusions of GR and MR antagonists were ineffective. While methodological differences might explain some of the discrepancies between these two studies, they cannot explain them all. It is therefore significant that intra-hippocampal microinfusion of spironolactone, an MR antagonist, produced clear, anxiolytic-like effects in the black-white transition model of anxiety, while a GR antagonist had no such effect (Smythe et al., 1997). Smythe et al. also provided evidence, using a short (10 min) or long (3 hr) injection-test interval, that the anxiolytic-like effects of spironolactone were mediated by non-

genomic mechanisms. This is because its anxiolytic effects occurred only at the short infusion-test interval, when genomic effects were unlikely (Haller et al., 2008). Neither antagonist produced anxiolysis at the long injection-test interval (Smythe et al., 1997).

The current study sought to extent these findings by examining the effects on fear and memory of infusing an MR antagonist (RU28318) into the dorsal hippocampus or ventral hippocampus. Based on the dissociations found in previous studies of the ventral and dorsal hippocampus (e.g., Bannerman et al., 2004; McEown and Treit, 2010), it seemed reasonable to expect that RU28318 infusions into the ventral hippocampus might impair unconditioned fear behaviour, while RU28318 infusions into the dorsal hippocampus could impair conditioned fear behaviour. These expectations were tested in two models of unconditioned fear or anxiety, the elevated plus-maze and the shock-probe burying tests. Conditioned fear effects were tested by imposing a 24 hr delay between electric shock in the burying chamber (acquisition) and subsequent behavioural testing in the same apparatus with a non-electrified probe (retention). Because the medial prefrontal cortex (mPFC) has also been implicated in fear and anxiety, and has extensive connections with the hippocampus, the effects of intra-mPFC infusions of RU28318 were also assessed in the same behavioral models.

Experiment 1:

Materials and methods

Subjects

Fourty-seven Sprauge-Dawley rats (Ellerslie, Edmonton, Alberta, Canada) were used. Each animal weighed between approximately 150-250 grams upon arrival. Food and water were available ad libitum. Animals were individually housed in polycarbonate cages and kept on a 12:12 hour light/dark cycle (lights on at 0700 hours). Behavioural testing occurred during the light portion of the cycle.

Surgery

All surgeries conformed to the Society for Neuroscience Guidelines, CCAC guidelines and to local animal care protocol # 6851004. Just prior to surgery, all subjects were injected with: 1) an analgesic (Rimadyl; 0.1cc, s.c.) to alleviate potential post-operative pain; 2) atropine sulfate (0.1mg/0.2ml, i.p.) to reduce any potential respiratory complications encountered during surgery; and 3) physiological saline to avoid dehydration (3cc, s.c., once before surgery). Subjects were anesthetised with Isoflurane gas (4% concentration in O_2 gas) and maintained at a 2% concentration throughout the duration of the surgery. Subjects were bilaterally implanted with 22-gauge, 8mm guide cannulae into the ventral hippocampus (n = 26; -5.2 mm AP, -5.7 mm DV, +/- 5.6 mm lateral to midline) and 5mm guide cannulae into the dorsal hippocampus (n = 21; -3.1 mm AP, - 3.3 mm DV, +/- 2.5 mm lateral to the midline). These anatomical coordinates were selected using a stereotaxic atlas (Paxinos and Watson, 1986). Two days after surgery, all cannulae were tested for obstruction by inserting dummy cannulae into each cannulae tract, and hibitane (an antibacterial/antifungal cream) was applied to the surgical area.

Elevated plus-maze

Upon arrival, rats were allowed three days to acclimatize to the colony room. After the acclimatization period all rats were individually handled for 5 minutes per day over a two day period. Surgeries were then performed with behavioural testing occurring six days post-surgery, during which time rats were handled daily during inspections for cannulae patency. The testing apparatus consisted of four elevated platforms (arms) of which two are open and two are enclosed by walls. Rats normally spend the majority of time in the closed arms, while avoiding the open arms. The amount of time spent in the open arms and the number of open-arm entries are used as measures of anxiety. Increases in these measures of open arm activity indicate anxiolysis. The total number of arm entries and the total number of closed-arm entries are common measures of general activity in the plus-maze.

Shock-probe burying test

A day after the plus-maze test, each rat was handled for 5 minutes over two days. Rats were then habituated to the shock-probe test apparatus for 15 min over 4 successive days, without the shock probe present. The apparatus consisted of a 40 × 30 × 40 cm Plexiglas chamber, with 5 cm of bedding material (wood chips) spread evenly over the chamber floor. The Plexiglas shock-probe (6.5 cm long 0.5 cm in diameter) was helically wrapped with 2 copper wires and inserted through a hole in one of the walls of the chamber, 2 cm above the bedding material. The probe was electrified using a two-pole (bipolar AC) current reversal "square wave" output (Model H13-15, Colbourne Instruments) set at 2mA. Rats

are then placed in the Plexiglas chamber, facing away from the electrified probe. During the 15 min test, rats typically receive one or two contact-induced shocks from the electrified probe, which normally elicits "burying behavior" in which they spray bedding material toward or over the probe, with rapid, alternating movements of the forepaws, while avoiding further contact with the shock-probe. A reduction in the duration of probe-burying, in the absence of a decrease in general activity or shock-sensitivity is used as the primary index of anxiety reduction. A reduction in simple passive avoidance of the probe can also be used as a measure of anxiety reduction in this test.

Infusion procedure

Rats were randomly assigned to either a control or experimental group. All microinfusions were bilateral. The control group received an intra-hippocampal infusion of physiological saline (infusion rate: 3µl/1 min for 1 minute; pH 7.4) and the experimental group received an intra-hippocampal infusion of RU28318 (Sigma) dissolved in artificial cerebral spinal fluid (Infusion rate: 3µl/1min for 1 minute) resulting in an infusion of 150ng of RU28318 per hemisphere. Infusions were administered 10 minutes prior to the acquisition session for all subjects. Infusions were administered with a 10 µl Hamilton microsyringe using an infusion pump (Harvard Apparatus 22). The cannulae were left in place for 30 seconds after drug administration to allow for diffusion.

Elevated plus-maze

Rats were tested in groups representing three conditions 1) RU28318 infusions into the dorsal hippocampus (n = 13), 2) RU28318 infusions into the

ventral hippocampus (n = 17) and 3) vehicle (saline) infusions into the dorsal or ventral hippocampus (n = 17). Testing began immediately after the animal was placed in the center of the maze and lasted 5 minutes. The number of arm entries (i.e., all four paws crossing into an arm) and the amount of time spent in each arm were recorded.

Acquisition – shock probe burying test

Rats were tested in groups representing four conditions 1) RU28318 infusions into the ventral hippocampus (n =8), 2) RU28318 infusions into the dorsal hippocampus (n = 8), 3) vehicle (saline) infusions into the dorsal or ventral hippocampus (n = 15). Each subject was individually placed into the chamber, facing away from the shock-probe. Testing began after rats' initial contact-induced probe-shock, and lasted 15 minutes. The shock probe was constantly electrified throughout the testing session. The number of contact-induced shocks, mean shock reactivity and still time (i.e., an inverse measure of general activity) was recorded. Mean shock reactivity was evaluated using a four point scale (see Pesold and Treit, 1992; for further detail).

Retention – shock probe burying test

After a 24-hour delay, rats were re-tested in the same chamber in which acquisition occurred, except that 1) the probe was not electrified, and 2) microinfusions were not administered. The amount of time rats spent in the half of the chamber farthest away from the shock-probe was taken as a measure of memory (see McEown and Treit, 2009; for further detail).

Histology

After the completion of behavioural testing, rats were deeply anesthetised using pentobarbital (Nembutal, 100 mg/kg, i.p) and subsequently perfused with a 10% formalin solution. Their brains were removed and placed in specimen jars containing a 10% formalin solution. After forty-eight hours, brains were sectioned (60 µm), stained with thionin, and mounted on microscope slides. The locations of cannulae were confirmed microscopically. The behavioural data for rats with either one or both cannulae outside of the target area (dorsal or ventral hippocampus) were omitted from analysis.

Statistical analyses

All control measures (e.g., general activity) were assessed with ANOVA (α = 0.05). A priori predictions (i.e., directional predictions made prior to data collection and analyses) were assessed using planned pair-wise comparisons (t-tests, $\alpha = 0.05$, two-tailed). Durations that rats buried the probe were transformed to their base 10 logs to correct for heterogeneity of variance.

Experiment 2:

Methods

Subjects

Thirty-one male Sprague-Dawley rats (Ellerslie, Edmonton, Alberta, Canada) were used. All other procedures were identical to experiment one.

Surgery

Surgical procedures were the same as those used in experiment one, with the exception that 31subjects were bilaterally implanted with 22-gauge, 5mm guide cannulae into the medial prefrontal cortex (n = 30; +2.9 mm AP, -1.8 mm

DV, +/- 1.9 mm lateral to the midline). These anatomical coordinates were selected using the stereotaxic atlas of Paxinos and Watson (1986). Post-operative care was identical to experiment one.

Infusion procedure

Rats were randomly assigned to either a control or experimental group. All infusions were bilateral. The control group (n=15) received an intra-mPFC infusion of physiological saline (infusion rate: 1µl/1 min for 1 minute; pH 7.4) and the experimental group (n=16) received an intra-mPFC infusion of RU28318 (Sigma) dissolved in artificial cerebral spinal fluid (Infusion rate: 1µl/1min for 1 minute) resulting in a 150ng of RU28318 per hemisphere. Infusions were administered 10 minutes prior to the acquisition session for all subjects. Infusions were administered with a 10 µl Hamilton microsyringe using an infusion pump (Harvard Apparatus 22). The cannulae were left in place for 30 seconds after drug administration to allow for diffusion.

The post-infusion behavioural testing procedures were the same as those used in experiment 1, as were the statistical and histological procedures.

Results

Experiment 1: MR antagonism in the dorsal and ventral hippocampus

Subjects and histology

Data for one rat could not be coded for the shock-probe burying test due to an error with the video equipment and data from another rat could not be collected for the shock-probe burying test as one of its cannulae was obstructed. Data from an additional three rats was discarded due to misplaced cannulae. Figures 2-1 and

2-2 show the bilateral placements of cannulae in the dorsal and ventral hippocampus, respectively.

Elevated plus-maze

Between-group differences in the control measures were non-significant, indicating that RU28318 did not affect locomotor activity (number of closed arm entries ($F(2, 44) = 1.94$, $p = .15$); total number of arm entries ($F(2, 44) = 2.32$, $p = .11$; see Table 2-1 for descriptive statistics). Figure 2-4 shows the means and standard errors of the experimental and control groups' open-arm activity in the elevated plus-maze. As predicted, rats microinfused with RU28318 into the ventral hippocampus spent a significantly greater percentage of time in the open arms ($t(32) = 2.07$, $p < .05$) compared to controls (Figure 2-4), whereas rats infused with RU28318 into the dorsal hippocampus were not significantly different from controls ($t(28) = .95$, $p = .35$). Unexpectedly, however, RU28318 infused into the ventral hippocampus did not significantly increase the percentage of open-arm entries ($t(32) = .126$, $p = .88$; for means and SEMs see Table 2-1). Although it is not uncommon to find significant effects for one percentage measure but not the other in the elevated plus-maze, the anxiolytic effects of RU28318 in the ventral hippocampus in this experiment were not particularly robust.

Shock probe burying test

Acquisition. Still time ($F(3, 20) = 1.37$, $p = .27$), number of shocks received ($F(3, 20) = .60$, $p = .55$) and shock reactivity ($F(2, 20) = 1.84$, $p = .18$) were all non-significant (see Table 2-1 for descriptive statistics) indicating that non-specific

drug effects on general activity and pain sensitivity do not explain the effects on anxiety. Ventral hippocampal microinfusions of RU28318 significantly decreased rats' burying behaviour compared to saline infused, shocked-controls ($t(13) = -3.74$, $p < .01$; see Fig. 2-5). Interestingly, dorsal hippocampal microinfusions of RU28318 also significantly reduced burying behaviour, compared to saline infused, shocked-controls ($t(13) = -3.29$, $p < .01$). Shock-naive rats did not engage in any burying behaviour during acquisition.

Retention. Shocked-control rats avoided the probe significantly more than shock-naive controls ($t(12) = -2.67$, $p < .05$), indicating that the shocked-control group retained a memory of the initial shock experience. However, antagonizing MRs in the dorsal or ventral hippocampus had no significant effect on memory of the initial shock experience (dorsal hippocampus RU28318 versus shocked-controls ($t(12) = .48$, $p = .63$); ventral hippocampus RU28318 versus shocked-controls ($t(13) = .43$, $p = .66$).

Experiment 2: MR antagonism in the mPFC

Subjects and histology

Four rats did not make contact with the shock-probe during the acquisition session. Data from one rat was discarded from the analysis of burying time because its score was more than three standard deviations from the mean. Data from an additional four rats was discarded due to misplaced cannulae. An additional rat could not be tested due to blocked cannulae. Figure 2-3 shows the correct bilateral placements in the mPFC.

Elevated plus-maze

The groups did not differ in terms of number of closed arm entries ($t(24) = 2.02$, $p = .06$) or total number of arm entries ($t(24) = 1.83$, $p = .08$; see Table 2-2 for descriptive statistics). RU28318 had no significant effect on percentage of open arm time ($t(24) = .52$, $p = .60$) or percentage of open arm entries ($t(24) = -.98$, $p = .33$), compared to saline infused controls (see Table 2-2 for descriptive statistics). These results suggest that MR receptors in mPFC do not mediate unconditioned anxiety in the plus-maze.

Shock probe burying test

Still time ($t(19) = 1.23$, $p = .23$), number of shocks ($t(19) = -1.23$, $p = .23$) and shock reactivity ($t(19) = 1.41$, $p = .18$) were all non-significant (see Table 2-2 for descriptive statistics), indicating that RU28318 did not affect locomotion or pain reactivity. In spite of the its null effects in the elevated plus-maze, however, RU28318 infused into the mPFC significantly impaired burying behavior compared to saline infused, shocked-controls ($t(19) = -2.49$, $p < .05$; see Fig. 2-6). These data suggest that unconditioned burying in the shock-probe test is sensitive to the anxiolytic-like effect of MR receptor antagonism in the medial prefrontal cortex.

Discussion

Antagonizing MRs in the dorsal hippocampus, ventral hippocampus or medial prefrontal cortex impaired unconditioned fear behaviour in the shock-probe burying test, but only ventral hippocampal MR antagonism impaired fear behavior in the elevated plus-maze test. In spite of the known roles of the hippocampus in conditioned fear and extinction, antagonist of MRs in the

hippocampus did not affect memory of the shock-probe. This experiment was the first to demonstrate that MRs in the ventral hippocampus and medial prefrontal cortex mediate unconditioned fear behaviour, and the first to explore the role of hippocampal MRs in memory of a shock experience.

Differences in inhibitory post-synaptic currents [IPSCs] in the dorsal and ventral hippocampus may be relevant to some of the results found in the present experiment. It has been found that MR antagonism in the ventral hippocampus increased IPSCs in this area, thereby inhibiting neuronal function. In contrast, MR antagonism in the dorsal hippocampus did not increase IPSPs, presumably allowing normal neuronal function in this region (Maggio and Segal, 2009). I previously found that direct inhibition of ventral hippocampal neuronal function with lidocaine or muscimol reduced anxiety, but inhibition of dorsal hippocampal neurons did not (see introduction). Taken together, these results might explain why antagonism of dorsal hippocampal MRs did not suppress unconditioned fear behaviour in the elevated plus-maze, whereas inhibition of ventral hippocampal MRs did.

Nevertheless, here I found evidence that both dorsal and ventral hippocampal MRs mediate unconditioned fear in the shock-probe burying test. Suppression of either dorsal or ventral hippocampal MRs decreased unconditioned burying (Experiment 1). These results are also consistent with Bitran et al. (1997), who found that 1 ng of RU28318 microinfused into the dorsal hippocampus suppressed burying behaviour, although doses marginally lower (0.2ng) or higher (5 ng) did not. Despite the fact that I did not find statistically

significant increases in open arm-activity after dorsal hippocampal microinfusion of RU28318, there was a trend in that direction (see Fig. 2-4), and Bitran (1997) showed that increases in open-arm activity were significant after dorsal hippocampal microinfusion of 1 ng RU28318. Finally, Smythe et al. (1997) found a significant anxiolytic effect in the light-dark test of anxiety when an MR antagonist was microinfused into the dorsal hippocampus, further supporting a role for the dorsal hippocampus in mediating the anxiolytic effects of MR antagonism.

Considering the neuroanatomical connections between the medial prefrontal cortex and the hippocampus, as well as other structures known to mediate anxiety (e.g., amygdala), our novel finding of anxiolytic effects of RU28318 in the mPFC may not be too surprising. Furthermore, intra-mPFC microinfusion of excitotoxins, or $GABA_A$ agonists, also disrupt mPFC neurotransmission, and produce clear-cut anxiolytic effects in the plus-maze and the shock-probe burying test (Shah et al., 2004 a,b; Shah and Treit, 2004).

There are a number of potential reasons why MR antagonism in the hippocampus did not affect rats' memory of the shock probe in the present experiments. Although lesion studies have shown that the hippocampus is essential for the normal performance of rats in a number of tasks (e.g., spatial memory) the role of hippocampal MRs in these tasks is not entirely clear. For example, while some studies find that MR antagonists impair spatial memory in rats (e.g., Oitzl and de Kloet, 1992; Yau et al., 1999), others have found no effect (Conrad et al., 1999; Khaksari et al., 2007). On the other hand, genetic studies

have shown that spatial memory is facilitated in rats that overexpress MRs (Lai et al., 2007), and impaired in rats that do not express MRs (Berger et al., 2006; Qiu et al., 2010). Despite these findings, it is unclear to what extent spatial memory plays a role in rats' retention of a previous shock-probe experience.

MR antagonists also impair rats' classically conditioned, contextual fear memory (Ninomiya et al., 2010), another "hippocampal-dependent" process. It is important to note, however, that contextual fear conditioning may not be relevant to rats' avoidance of the shock-probe during the retention test in the present experiments, for two reasons. First, shock-probe avoidance is a simple, instrumental behavior, and recent evidence suggests that the brain mechanisms of instrumental fear conditioning may be quite different than the brain mechanisms of classical fear conditioning (Cohen and Castro-Alamancos, 2010). Second, the nature of what is learned in classical and instrumental fear conditioning is distinct. Classical conditioning depends on the acquisition of associations between conditioned and unconditioned stimuli, whereas instrumental conditioning depends on acquisition of associations between behavior and its consequences. Furthermore, instrumental avoidance of fear-inducing stimuli (e.g., the shock-probe) is under the complete control of the rat, whereas Pavlovian (CS) - (US) pairings (e.g., shock; tone), are under the control of the experimenter (Maren, 2003). Accordingly, direct comparisons between the effects of MR antagonists in the current study and effects found with contextual fear conditioning may not be particularly useful. While muscimol inactivation studies suggest that acquisition of Pavlovian fear conditioning can be dissociated along the dorsal-ventral axis of

the hippocampus (e.g., Maren and Holt, 2004), excitotoxic lesions along this axis produced more complex results not easily encompassed by a simple, functional dichotomy (e.g., Hunsaker and Kesner, 2008).

Finally, MRs mediate both slow acting genomic changes and fast acting non-genomic changes (see Joels, 2008; Haller et al., 2008). Acute MR antagonism (e.g., occurring once just ten minutes prior to testing in the current study) may have markedly different effects on unconditioned burying behavior and probe-avoidance than more chronic MR antagonism, which could induce the longer-term, genomic changes needed to consolidate learning. Indeed, the failure of MR antagonists to impair memory of a shock probe experience 24 hrs later may be explained by the fact that genomic changes may have been prevented by the short, 10 min interval between infusion and testing (Haller et al., 2008).

In summary, this study was the first to examine the separate roles of MRs in the dorsal hippocampus, ventral hippocampus and medial prefrontal cortex on fear and memory. I demonstrate for the first time that ventral hippocampus and medial prefrontal cortex MRs mediate unconditioned fear behaviour. On the other hand, I was not able to demonstrate that dorsal hippocampal MRs mediate memory of a shock-probe experience. Perhaps more significantly, I provide evidence that dorsal hippocampal MRs are involved in unconditioned defensive burying, which replicates earlier studies. These results provide further neuroanatomical insights into the complex role that corticosteroids perform in fear and memory.

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Experiment one

Plus-maze	Dorsal hippocampus (*n* = 13)	Ventral hippocampus (*n* = 17)	Controls (*n* = 17)	
Closed arm entries	7.15 (.96)	9.65 (1.03)	9.18 (.68)	
Total arm entries	10.92 (1.39)	14.24 (1.16)	13.24 (.73)	
% Open arm entries	34.97 (5.73)	32.55 (4.93)	31.64 (3.29)	
Shock-probe	**(*n* = 8)**	**(*n* = 8)**	**Shocked controls (*n* = 7)**	**Naïve controls (*n* = 7)**
Still time	202.75 (65.82)	117.63 (55.46)	38.86 (24.54)	111.86 (70.31)
Shock number	1.75 (.25)	1.50 (.18)	1.43 (.20)	NA
Shock reactivity	1.45 (.24)	1.25 (.25)	1.85 (.14)	NA

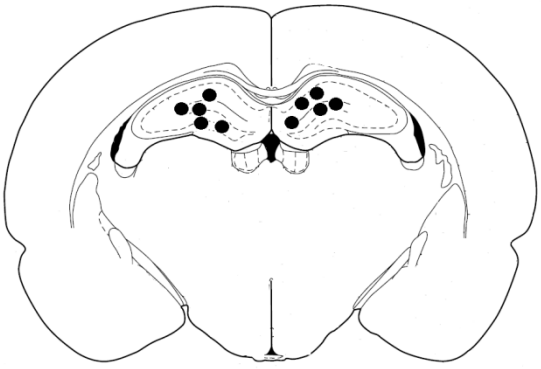
Table 2-1: Mean (± S.E.M.): 1) number of closed arm entries, 2) number of total arm entries, 3) percentage of open arm entries, 4) still time, 5) number of shocks, and 6) shock reactivity for Experiment one.

Experiment two

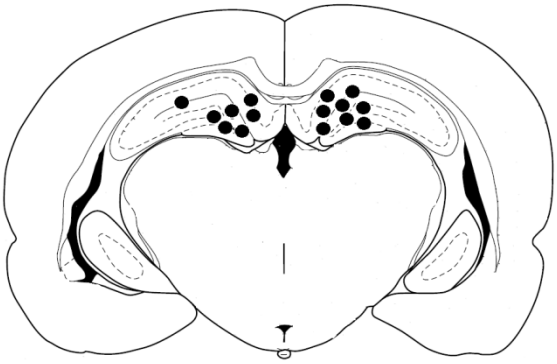
Plus-maze	Medial prefrontal cortex (n = 16)	Controls (n = 10)
Closed arm entries	8.56 (.78)	6.00 (1.0)
Total arm entries	12.31 (.86)	9.60 (1.24)
% Open arm time	16.93 (3.29)	14.47 (2.62)
% Open arm entries	32.37 (4.36)	38.97 (4.76)
Shock-probe	(n = 14)	(n = 7)
Still time	102.64 (38.61)	29.71 (29.71)
Shock number	2.00 (.27)	3.00 (1.02)
Shock reactivity	1.52 (.12)	1.22 (.15)

Table 2-2: Mean (± S.E.M.): 1) number of closed arm entries, 2) number of total arm entries, 3) percentage of open arm time, 4) percentage of open arm entries, 5) still time, 6) number of shocks and 7) shock reactivity for Experiment two.

Figure 2-1: Illustrations of coronal sections of the rat brain adapted from Paxinos and Watson (1986) displaying the approximate location of dorsal hippocampal infusion sites in Experiment 1. The numbers indicate A–P coordinates relative to bregma.



AP -3.30 mm bregma



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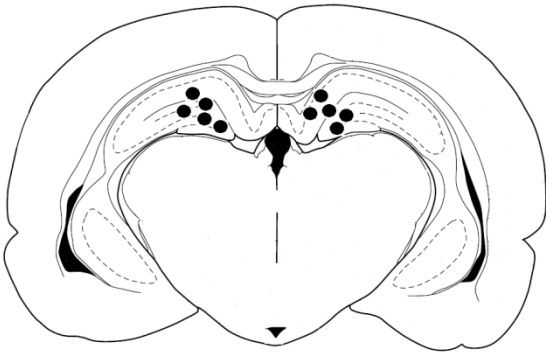
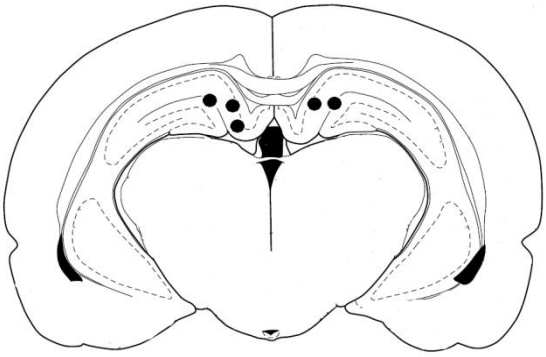
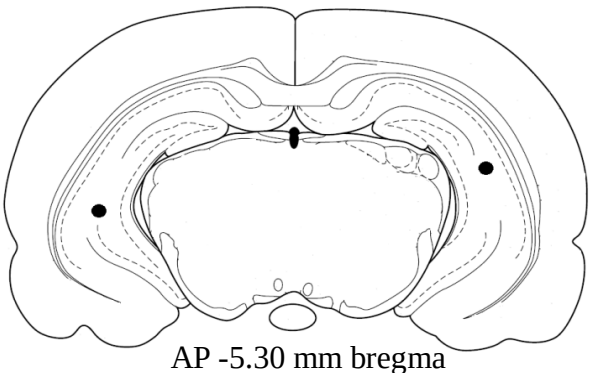


AP -4.30 mm bregma

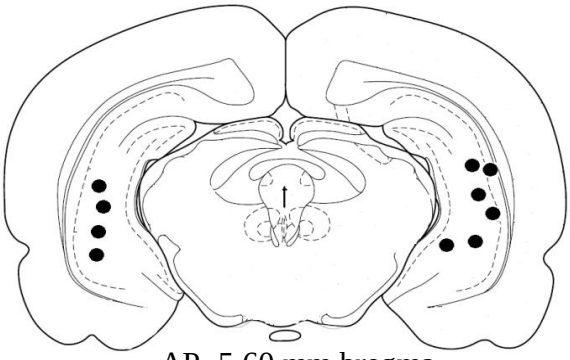


AP -4.52 mm bregma

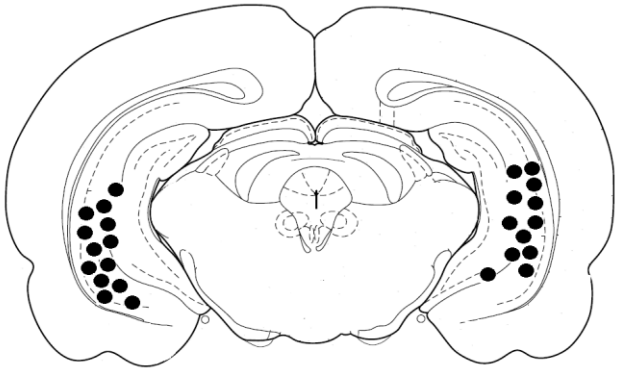
Figure 2-2: Illustrations of coronal sections of the rat brain adapted from Paxinos and Watson (1986) displaying the approximate location of ventral hippocampal infusion sites in Experiment 1. The numbers indicate A–P coordinates relative to bregma.



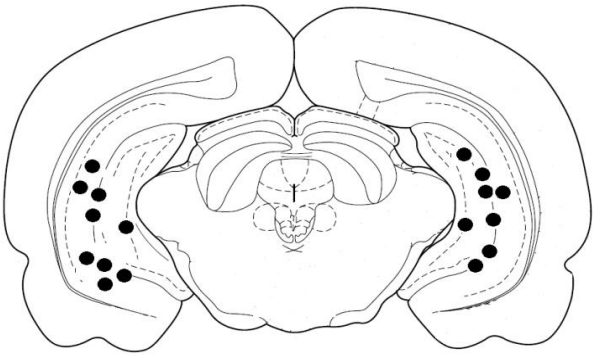
AP -5.30 mm bregma



AP -5.60 mm bregma

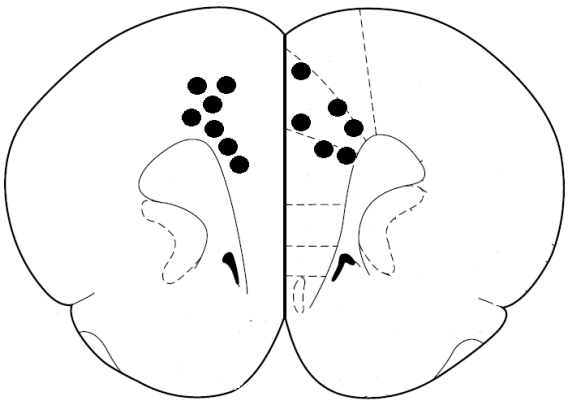


AP -5.80 mm bregma

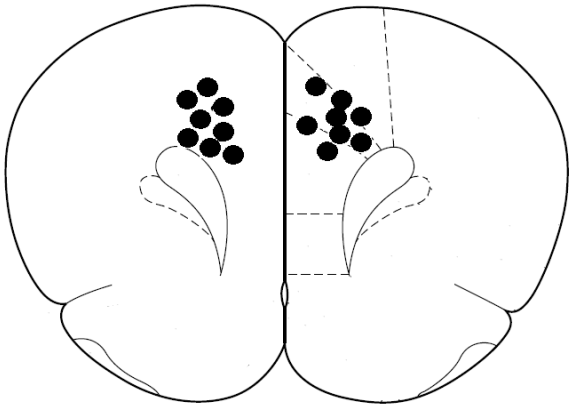


AP -6.04 mm bregma

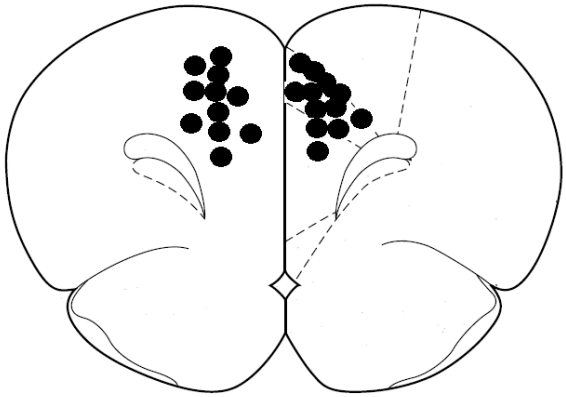
Figure 2-3: Illustrations of coronal sections of the rat brain adapted from Paxinos and Watson (1986) displaying the approximate location of medial prefrontal cortex infusion sites in Experiment 2. The numbers indicate A–P coordinates relative to bregma.



AP +2.70 mm bregma



AP +3.20 mm bregma



AP +3.70 mm bregma

Figure 2-4: Mean (±S.E.M.) percentage of open arm time (experiment one) of dorsal hippocampus (RU28318), ventral hippocampus (RU28318) and control (vehicle) rats.

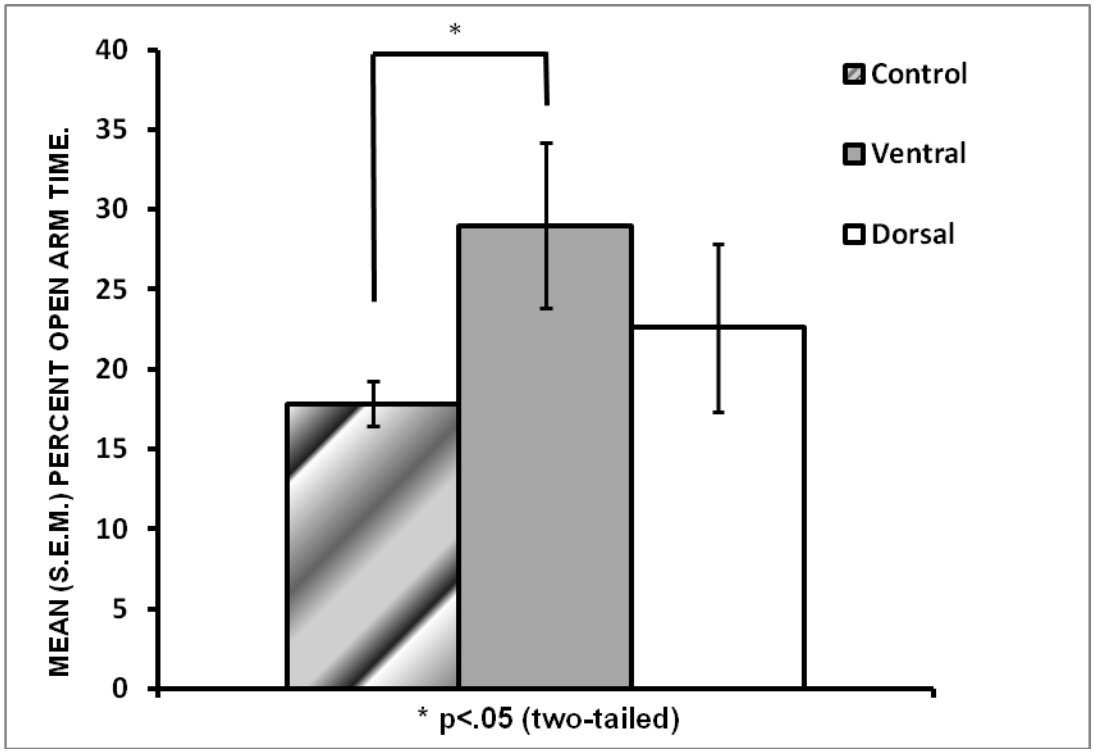

*
40
35
30
25
20
15
10
5
0
MEAN (S.E.M.) PERCENT OPEN ARM TIME.
Control
Ventral
Dorsal
* p<.05 (two-tailed)

Figure 2-5: Mean (±S.E.M.) bury time (experiment one) of dorsal hippocampus (RU28318), ventral hippocampus (RU28318) and control (vehicle) rats.

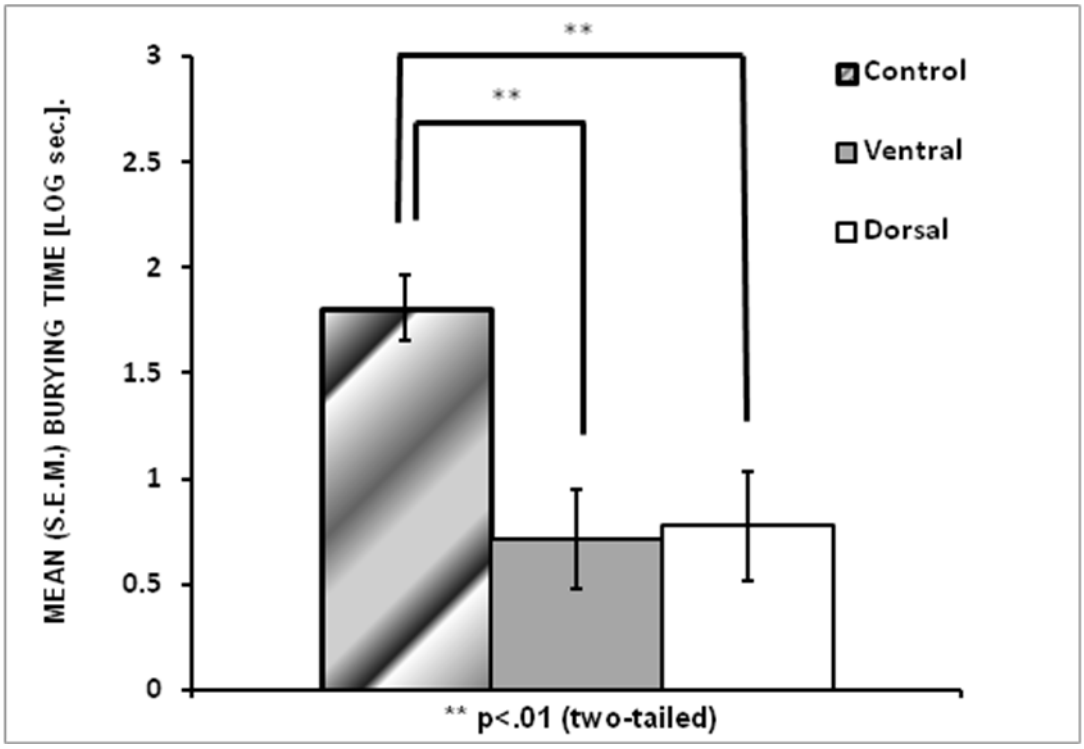
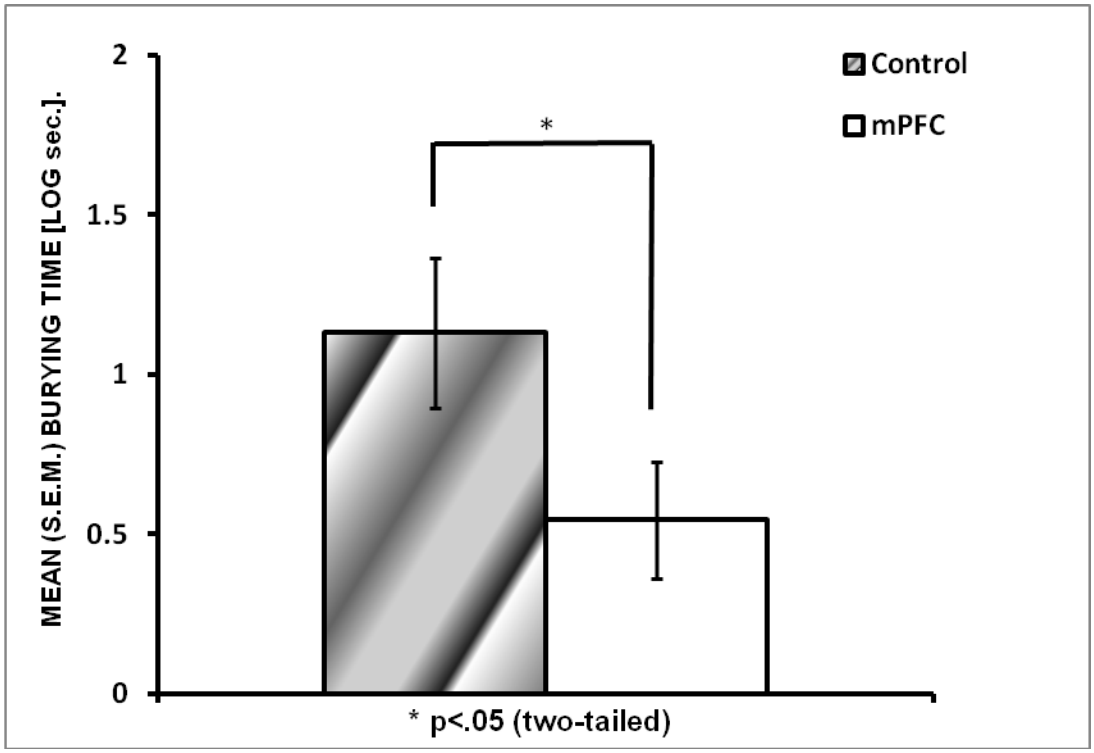

**
**
3
2.5
2
1.5
1
0.5
0
MEAN (S.E.M.) BURYING TIME [LOG sec.].
Control
Ventral
Dorsal
** p<.01 (two-tailed)

Figure 2-6: Mean (±S.E.M.) bury time (experiment two) of medial prefrontal cortex (RU28318) and control (vehicle) rats.


Control
mPFC
MEAN (S.E.M.) BURYING TIME [LOG sec.].
2
1.5
1
0.5
0
*
* p<.05 (two-tailed)

Chapter 3

The Role of Dorsal and Ventral Hippocampal $GABA_A$ Receptors in Mediating Anxiety

Introduction

$GABA_A$ receptors are ionotropic and consist of five protein sub-units. Although nineteen different $GABA_A$ sub-units exist (i.e., α1-6, β1-3; γ1-3; ρ1-3; θ; ε; π; and δ), the most frequently occurring $GABA_A$ receptor sub-unit combinations include two alpha (α1, α2, α3, or α5) two beta (β2 or β3) and one gamma (γ2) sub-unit (Uusi-Oukari and Korpi 2010). Benzodiazepines bind to $GABA_A$ receptors at the interface between alpha (e.g., α1, α2, α3, or α5) and gamma sub-units (γ2) (Gavish and Snyder 1980; Uusi-Oukari and Korpi 2010). When benzodiazepines bind to this allosteric site on the $GABA_A$ receptor they increase the frequency of $GABA_A$ receptor chloride channel openings normally produced by the endogenous neurotransmitter γ-aminobutyric acid (GABA) (Sigel and Steinmann 2012; Study and Barker 1981). This potentiation of neuronal inhibition produced by benzodiazepines is thought to mediate their anxiolytic effects (Korpi and Sinkkonen 2006).

Inverse agonists of the benzodiazepine receptor, such as FG-7142, also bind at the interface of the alpha (α1, α2, α3, or α5) and gamma (γ2) sub-units of the $GABA_A$ receptor (Braestrup et al. 1980). Furthermore, FG-7142 binds with higher affinity to α1 sub-units compared to α2, α3, or α5 sub-units (Atack et al. 2005; Evans and Lowry 2007). Importantly, FG-7142 *decreases* the frequency of chloride channel openings thereby *reducing* neuronal inhibition (Evans and Lowry 2007). Not surprisingly, inverse agonists such as FG-7142 typically produce behavioral effects opposite to those of benzodiazepine receptor agonists. For example, FG-7142 and other inverse agonists increase anxiety, in both

animals and humans, and these anxiogenic effects can be reversed with benzodiazepine receptor antagonists such as flumazenil (Atack et al. 2005; Cole et al. 1995; Dawson et al. 2006; Dorow et al. 1983; Dorow 1987; Horowski and Dorow 2002; Pellow and File 1986; Pellow et al. 1987; Rogers et al. 1995; Sink et al. 2010; Waters and See 2011).

A large body of experimental and clinical evidence now suggests that the benzodiazepine binding site on the $GABA_A$ receptor represents an important molecular mechanism for the control of anxiety (e.g., Korpi and Sinkkonen 2006). Benzodiazepine receptors are found throughout the brain but are highly concentrated in structures previously implicated in anxiety (Young and Chu 1990; Zezula et al. 1988). For example, the hippocampus contains $GABA_A$ receptors with allosteric binding sites for benzodiazepines and other anxiolytic drugs (Sotiriou et al. 2005; Sarantis et al. 2008). Furthermore, a number of studies have shown that benzodiazepines microinfused into the hippocampus produce anxiolytic effects in animal models such as the Vogel conflict test and elevated plus-maze test (Gonzalez et al. 1998; Kataoka et al. 1991; Menard and Treit 2001; Plaznik et al. 1994; Stefanski et al. 1993). However, benzodiazepine microinfusions were restricted to dorsal regions of the hippocampus in most of these studies, and there is evidence to suggest that ventral regions of the hippocampus also contain $GABA_A$/benzodiazepine receptors (Sarantis et al. 2008) and may be more important for the control of anxiety than the dorsal region (e.g., Fanselow and Dong 2010). For example, studies have found that lesioning the ventral hippocampus produces anxiolysis in animal models of anxiety (e.g.,

Kjelstrup et al. 2002; McHugh et al. 2004). In addition, infusing the $GABA_A$ agonist muscimol into the ventral hippocampus produced anxiolysis in the shock-probe burying test (McEown and Treit 2010) as did temporary sodium channel blockade (McEown and Treit 2009; Degroot and Treit 2004). Taken together, I would expect that infusing a benzodiazepine (i.e., diazepam) into the ventral hippocampus should produce anxiolysis in an animal model of anxiety such as the elevated plus-maze.

Based on evidence of the anxiogenic effects of inverse agonists such as FG-7142, I would also expect that infusing FG-7142 into the ventral or dorsal hippocampus would produce *anxiogenesis* in the elevated plus-maze. Finally, the results of these experiments will provide a broader molecular context from which to view later studies in this thesis, where I examine the effects of intra-hippocampal infusion of selective ligands of individual sub-units of the $GABA_A$ receptor in the same animal models of anxiety.

Methods

Subjects

Thirty one Sprauge-Dawley rats obtained from Ellerslie (Edmonton, Canada) were used for the experiment. Each rat weighed between 150-250 grams when they arrived. Water and food were available to rats without restriction. Rats were tested during the light portion of a 12:12 hour light/dark cycle.

Surgery

All animal surgeries were performed under guidelines established by the Society for Neuroscience, Canadian Council for Animal Care and a local animal care

protocol. Animals were randomly assigned to receive dorsal or ventral hippocampus cannulae implants. Prior to surgery rats were injected with an analgesic (Rimadyl; 0.05cc, s.c.) to avoid post-operative pain and saline (3cc, s.c., once before surgery) to avoid dehydration during and after surgery. Isoflourane gas was used to anesthetize rats (4% concentration in O_2 gas) and to maintain anaesthesia during surgery (2% concentration in O_2 gas). Rats were then implanted with 22-gauge, 5mm guide cannulae into the dorsal hippocampus (-3.1 mm AP, - 3.3 mm DV, +/- 2.5 mm lateral to the midline) and 8mm guide cannulae into the ventral hippocampus (-5.2 mm AP, -5.7 mm DV, +/- 5.6 mm lateral to midline). These coordinates were chosen using a stereotaxic atlas (Paxinos and Watson 1986). Two days following surgery an antibacterial cream was re-applied to the surgical area to prevent infection and cannulae were inspected for obstruction by inserting dummy cannulae into each cannulae tract. Behavioural testing occurred six days after surgery.

Elevated plus-maze

The plus-maze consists of four elevated arms of which two are open (measuring 50 x 10 cm) and two are enclosed by walls (measuring 50 x 10 x 50 cm). Rats prefer to spend the majority of time within the enclosed arms, avoiding the open arms. The percentage of time spent in the open arms (open arm time divided by total arm time multiplied by 100) and the percentage of entries made into the open arms (number of open arm entries divided by total number of arm entries multiplied by 100) are used as measures of anxiety in this test (Pellow and File 1986). Increases in either of these indexes indicate anxiolysis. The total number of

entries into any arm and the total number of entries into the enclosed arms serve as measures of general activity in the plus-maze. The testing period was five minutes in duration and the behaviour of each rat was video-taped during the testing period for later analysis.

Infusion procedure

Rats were randomly assigned to vehicle or drug conditions. All microinfusions were bilateral. The vehicle control group (n = 10) received hippocampal infusions of 10% DMSO in physiological saline (infusion rate: 1µl/1 min for 1 minute; pH 7.4). The two experimental groups received hippocampal infusions of either diazepam (Sabex) or FG-7142 (Tocris). Diazepam was dissolved in 40% propylene glycol, 10% dehydrated alcohol, 4.25% benzoic acid, and 1.5% benzyl alcohol, to a final concentration of 5mg/ml (infusion rate 2 µl/min for 1 min; pH 7.4). Each rat in this group (n =10) received 10µg/µl of diazepam per hemisphere. FG-7142 was in powdered form, and was mixed in a solution of 10% DMSO and saline to a final concentration of 10µg/µl (infusion rate 1 µl/min for 1 min; pH 7.4.). When administered peripherally in rats, 10mg/kg is the minimally effective dose of FG-7142 that affects anxiety (Cole et al. 1995). Based on these data, a 10µg/µl dose was selected for direct infusion into the hippocampus. Rats in this group (n = 10) received 10µg/µl of FG-7142 per hemisphere. Diazepam and FG-7142 were infused using a 10µl Hamilton microsyringe and an infusion pump (Harvard Apparatus 22). The cannulae needles remained in their tracts for 30 seconds after administering each drug to allow the compounds to properly diffuse. All infusions occurred ten minutes before testing.

Histology

Following the completion of behavioural testing, rats were anesthetized using Isoflourane gas (5% concentration in O_2). Rats were then perfused with a 10% formalin solution. After perfusions were complete their brains were removed and placed into jars containing a 10% formalin solution. Forty eight hours later, brains were removed from the jars and sectioned at a thickness of 60µm. Brains were then stained using thionin and mounted on microscope slides for inspection of cannulae placement using a light microscope. Rats with one or both cannulae outside the target brain areas (i.e., the dorsal or ventral hippocampus) were removed from statistical analysis of behaviour.

Statistical analyses

ANOVA was used to assess control measures for general activity (i.e., number of closed arm entries and total number of arm entries) followed where necessary with post-hoc, pair wise comparisons ($\alpha = 0.05$). Anxiety measures (i.e., percentage of open-arm time and percentage of open arm entries) were assessed using individual, a priori, pair-wise comparisons (t-tests, $\alpha = 0.05$, two-tailed).

Results

Histology

Data from one rat was excluded from the final analyses due to misplaced cannulae. After excluding this data, 30 rats were left in the experiment [dorsal hippocampus diazepam infusions = 4; ventral hippocampus diazepam infusions = 6; dorsal hippocampus FG-7142 infusions = 5; ventral hippocampus FG-7142

infusions = 5 and vehicle infusions = 10]. Figure 3-1 shows the correct bilateral placements of cannulae in the dorsal and ventral hippocampus.

Elevated plus-maze

Control measures were non-significant between groups (number of closed arm entries: $F(4, 25) = .61$, $p = .65$; number of total arm entries: $F(2, 25) = 1.35$, $p = .27$) indicating that locomotor activity was not affected by diazepam or FG-7142 (see Table 3-1 for descriptive statistics).

Rats infused with diazepam into the ventral hippocampus made proportionally more entries into the open arms than did vehicle-infused controls ($t(14) = 2.36$, $p<.05$) (see Figure 3-2). In addition, diazepam increased the percentage of time they spent in the open arms compared to vehicle infused controls ($t(14) = 2.07$, $p<.05$) (see Figure 3-3).

Rats infused with diazepam into the dorsal hippocampus also made proportionally more entries into the open arms compared to vehicle-infused controls ($t(13) = 2.66$, $p<.01$) (see Figure 3-2). On the other hand, diazepam in the dorsal hippocampus did not increase the percentage of time they spent in the open arms, compared to controls ($t(13) = 1.38$, $p = .18$) (see Figure 3-3). Overall, these results suggest that diazepam produces anxiolysis when infused into either the dorsal or the ventral hippocampus.

In contrast to the expectation of anxiogenesis, FG-7142 infusions into the dorsal or ventral hippocampus did not significantly affect anxiety in the plus-maze, compared to vehicle-infused controls (% entries: dorsal hippocampus: $t(13) = 1.32$, $p = .20$; ventral hippocampus: $t(13) = .64$, $p = .53$; % time: dorsal

hippocampus: t(13) = -.14, p = .88; ventral hippocampus t(13) = - .19, p = .85) (see Figure 3-4).

Discussion

Results from this study are the first to suggest that the anxiolytic effect of benzodiazepines is partially mediated by $GABA_A$ receptors in the ventral hippocampus. These findings also replicate previous research in which direct and indirect agonists of $GABA_A$ receptors in the dorsal hippocampus produced anxiolysis in the elevated plus-maze and in other tests of unconditioned anxiety. In contrast, I found that FG-7142, an inverse agonist at the benzodiazepine receptor site, did not affect anxiety in either the dorsal or ventral hippocampus.

The ventral hippocampus has extensive connections with areas of the brain that are thought to mediate anxiety such as the septum and amygdala, which also contain $GABA_A$/benzodiazepine receptors (Risold and Swanson 1997; Petrovich et al. 2001; Pitkanen et al. 2000; Onoe et al. 1996). Infusing benzodiazepine anxiolytics into the septum or amygdala also produce anxiolysis the elevated plus-maze and other tests of unconditioned anxiety (e.g., Green and Vale 1992; Pesold and Treit 1994, 1996; Zangrossi and Graeff 1994). Recent evidence using the direct $GABA_A$ agonist muscimol suggests that ventral hippocampal $GABA_A$ receptors and lateral septal $GABA_A$ receptors work in concert to mediate anxiety (Trent and Menard 2010). The ventral hippocampus may play a central role within a network of interconnected brain structures that mediate anxiety through activating $GABA_A$ benzodiazepine receptors.

Be this as it may, I also found that dorsal hippocampal diazepam infusions produced significant anxiolysis in the plus-maze, at least with one of the measures of anxiety. These positive results are consistent with several other studies (Menard and Treit 1999; Engin and Treit 2007; Plaznik et al. 1994; Menard and Treit 2001; Rezayat et al. 2005). In contrast, still other studies have found that dorsal hippocampal benzodiazepine infusions have inconsistent or null effects on anxiety in animal models (e.g., McNamara and Skelton 1993; Gonzalez et al. 1998; Petit-Demouliere et al. 2009). Methodological limitations, such as inadequate doses, questionable behavioral tests, and incomplete dorsal hippocampal infusions detract from the importance of these negative findings, however.

I predicted that intra-hippocampal FG-7142, an inverse $GABA_A$ agonist, would produce anxiogenesis in the elevated plus-maze. However, in spite of its well-documented anxiogenic effects, I found that FG-7142 had no effect on anxiety in the elevated plus-maze, regardless of whether it was infused into the dorsal or ventral hippocampus. There are other studies, however, that have infused FG-7142 directly into the brain and found inconsistent and sometimes contradictory effects on anxiety. For example, intracerebral infusions of FG-7142 have resulted in anxiogenic effects (Sena et al. 2003; Russo et al. 1993), anxiolytic effects (Sena et al. 2003) or null effects on anxiety (Russo et al. 1993). These contradictory effects to some extent lessen the failure here to obtain anxiogenic effects after infusions of FG-7142 into the dorsal hippocampus.

Nevertheless, the null effect of FG-7142 found here presumably occurred at exactly the same allosteric site where clear-cut anxiolytic effects were found with diazepam, and where the anxiogenic effects of FG-7142 are also thought to be mediated. Indeed, neurophysiological data comparing the effects of diazepam and FG-7142 on $GABA_A$ receptor function suggest that FG-7142 should have produced anxiogenesis when infused into either the dorsal or ventral hippocampus. $GABA_A$ $\alpha 2$ receptor sub-units, to which both FG-7142 and diazepam bind, are thought to mediate the effects of benzodiazepine agonists and inverse agonists on anxiety (e.g., Atack 2008; Low et al. 2000). Both diazepam and FG-7142 produce significant but opposite effects on chloride channel flux that are of comparable magnitude (see table 3 in Dawson et al. 2006, page 1340). The negative efficacy of FG-7142 on chloride ion flux, however, was not apparent at the behavioral level in the present experiments, as there was no evidence that intra-hippocampal infusions of FG-7242 produced anxiogenesis under any condition.

To summarize, in the current study I examined the role of dorsal and ventral hippocampus $GABA_A$ benzodiazepine receptors in mediating anxiety. I found that activating $GABA_A$ receptors in the dorsal or ventral hippocampus, using the benzodiazepine diazepam, reduced anxiety in the elevated plus-maze. However, inverse agonism of $GABA_A$ receptors, using FG-7142, had no effect on anxiety. Together these results suggest that while the anxiolytic effects of benzodiazepines are partially mediated through $GABA_A$ benzodiazepine receptors in the dorsal and ventral hippocampus, the anxiogenic effects of inverse agonists

of the benzodiazepine receptor site are not. Because there is a paucity of data on the effects of intracerebrally infused inverse agonists, their effects in other areas of the brain known to be involved in anxiety (e.g., amygdala) need systematic examination.

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		Diazepam		FG-7142	
	Controls	Dorsal hippocampus	Ventral hippocampus	Dorsal hippocampus	Ventral hippocampus
Plus-maze	*(n = 10)*	*(n = 4)*	*(n = 6)*	*(n = 5)*	*(n =5)*
Closed arm entries	7.60 (.94)	6.25 (1.65)	6.50 (.71)	8.60 (2.33)	6.00 (.89)
Total arm entries	9.90 (1.10)	13.50 (1.32)	13.83 (3.14)	11.80 (2.65)	8.20 (.86)
% Open arm entries	23.64 (3.28)	53.49 (10.78)	44.75 (10.33)	31.72 (5.67)	27.48 (5.32)

Table 3-1: Mean (± S.E.M.): 1) closed arm entries, 2) total arm entries, and 3) percentage of open arm entries.

Figure 3-1: Illustrations of coronal sections of the rat brain adapted from Paxinos and Watson (1986) displaying the approximate location of dorsal and ventral hippocampal infusion sites in Experiments 1 and 2. The numbers indicate A–P coordinates relative to bregma.

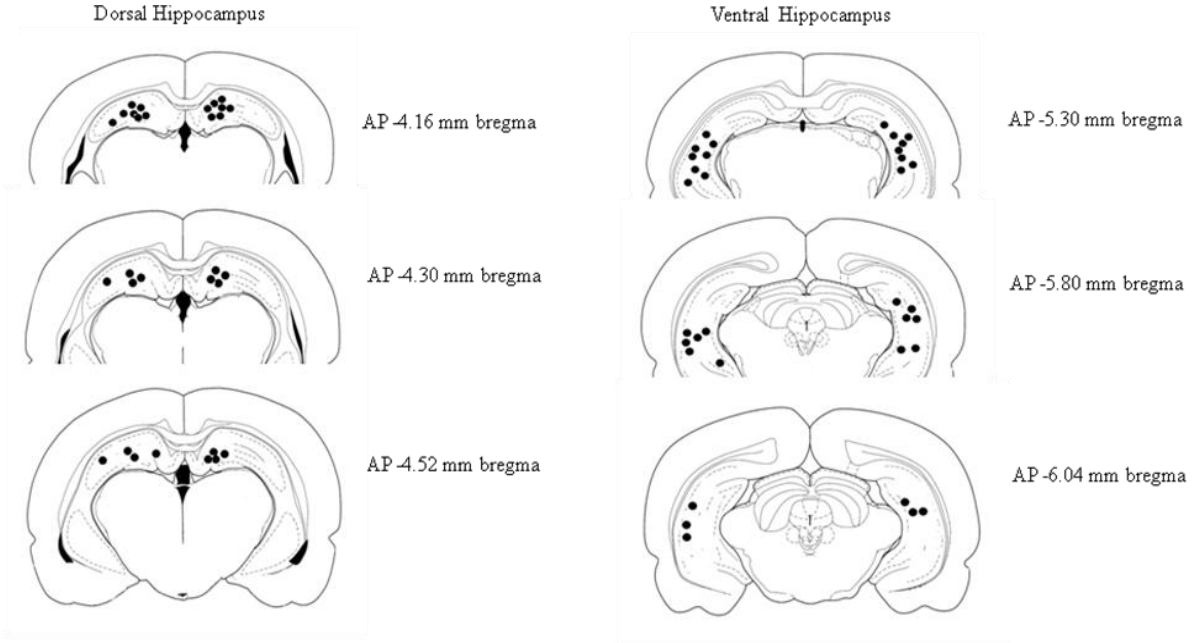

Dorsal Hippocampus
Ventral Hippocampus
AP -4.16 mm bregma
AP -4.30 mm bregma
AP -4.52 mm bregma
AP -5.30 mm bregma
AP -5.80 mm bregma
AP -6.04 mm bregma

Figure 3-2: Mean (± S.E.M.) percentage of open arm entries of dorsal hippocampus (diazepam), ventral hippocampus (diazepam) and control (vehicle) rats.

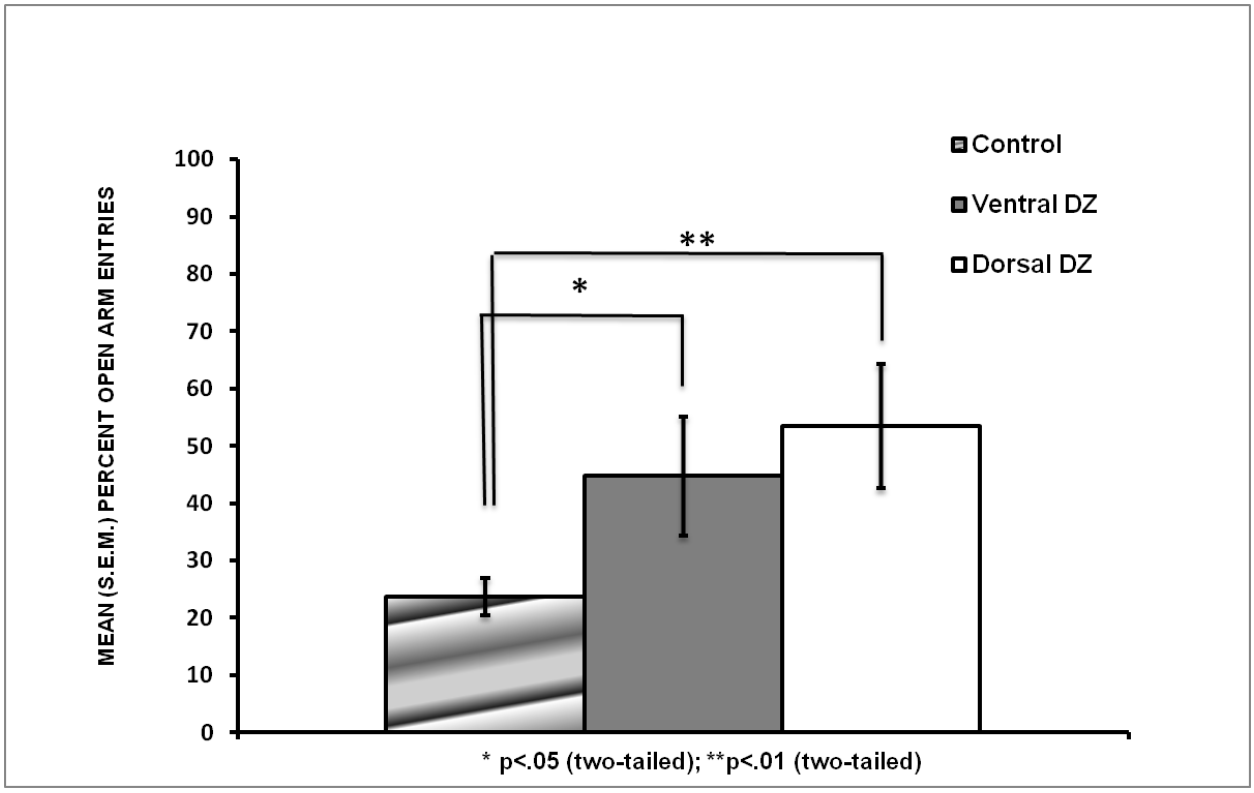

MEAN (S.E.M.) PERCENT OPEN ARM ENTRIES
100
90
80
70
60
50
40
30
20
10
0
**
*
Control
Ventral DZ
Dorsal DZ
* p<.05 (two-tailed); **p<.01 (two-tailed)

Figure 3-3: Mean (± S.E.M.) percentage of open arm time of dorsal hippocampus (diazepam), ventral hippocampus (diazepam) and control (vehicle) rats.

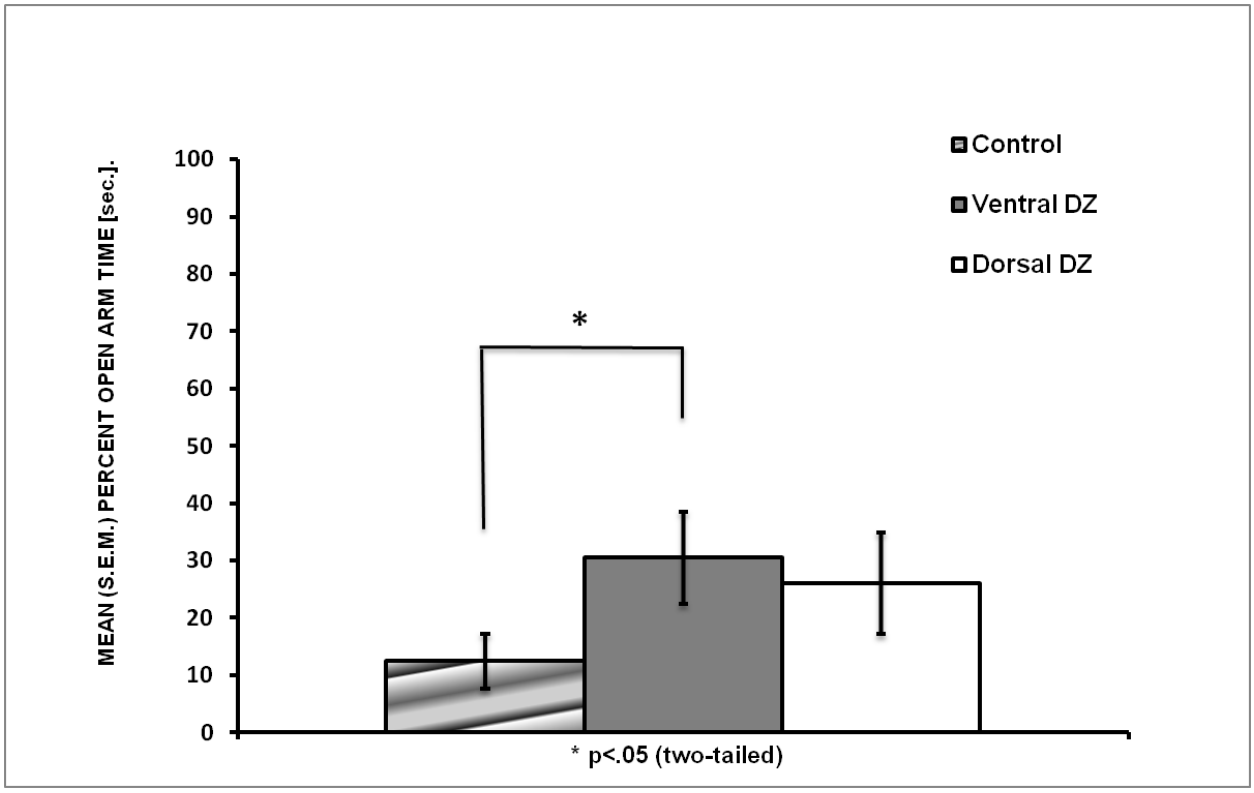
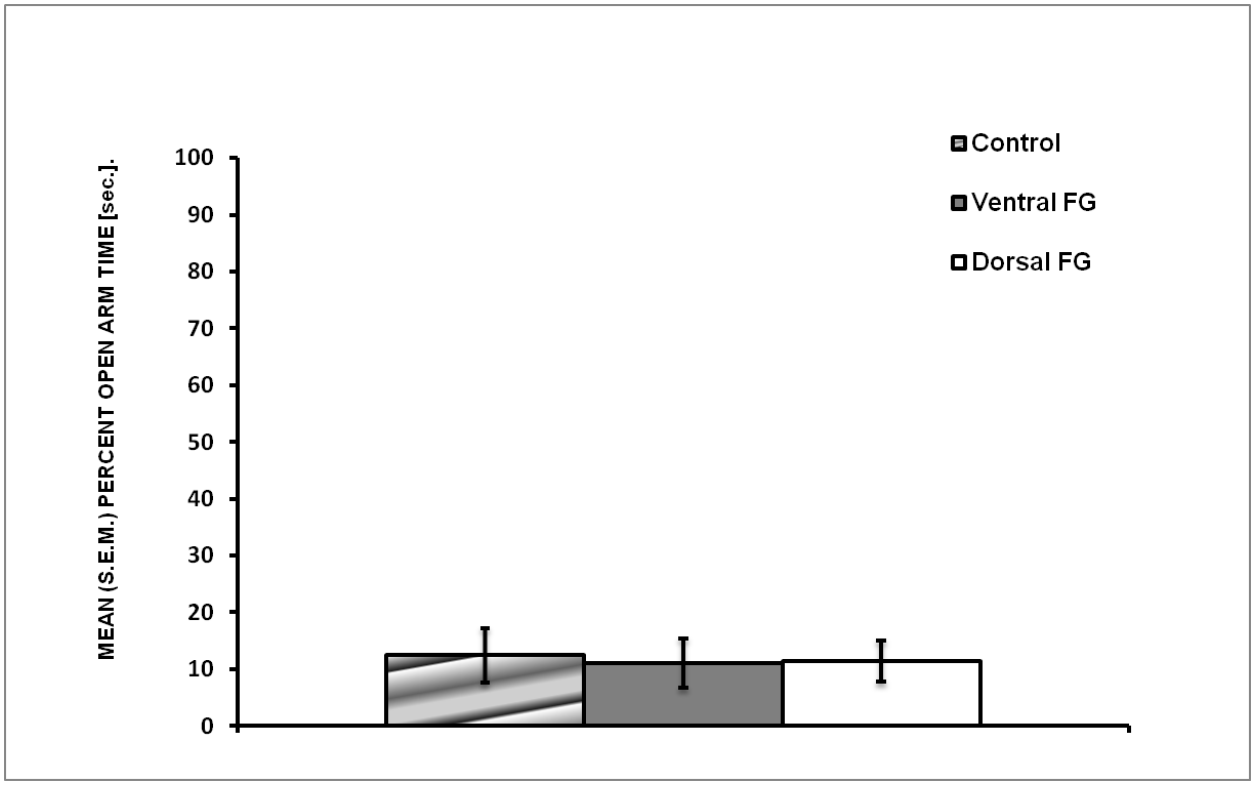

MEAN (S.E.M.) PERCENT OPEN ARM TIME [sec.].
100
90
80
70
60
50
40
30
20
10
0
*
Control
Ventral DZ
Dorsal DZ
* p<.05 (two-tailed)

Figure 3-4: Mean (± S.E.M.) percentage of open arm time of dorsal hippocampus (FG-7142), ventral hippocampus (FG-7142) and control (vehicle) rats.


MEAN (S.E.M.) PERCENT OPEN ARM TIME [sec.].
100
90
80
70
60
50
40
30
20
10
0
Control
Ventral FG
Dorsal FG

Chapter 4

α2 $GABA_A$ Receptor Sub-units in the Ventral Hippocampus and α5 $GABA_A$ Receptor Sub-units in the Dorsal Hippocampus Mediate Anxiety and Fear Memory.

A version of this chapter was submitted for publication in *Psychopharmacology*

Introduction

Diazepam and other benzodiazepines are positive allosteric modulators of the $GABA_A$ receptor, increasing the inhibitory effects of GABA, which presumably mediates their anxiolytic effects. In contrast, negative allosteric modulators of the $GABA_A$ receptor (e.g., FG-7142) *decrease* the inhibitory actions of GABA, producing effects opposite to that of benzodiazepines such as diazepam (e.g., increased anxiety). However, the individual and combined roles of specific $GABA_A$ receptor sub-units in the effects of positive and negative allosteric modulators of the $GABA_A$ receptor continue to be actively explored (e.g., Smith et al. 2012; Marowsky et al. 2012).

In order to characterize the functional roles of individual sub-units of the $GABA_A$ receptor in anxiety, researchers first disabled specific $GABA_A$ receptor sub-units by inserting mutations of these sub-units into the mouse genome and observing the effects in behavioral models of rodent anxiety (e.g., the elevated plus-maze, social interaction; McKernan et al. 2000; Low et al. 2000). These early "knock-out" studies indicated that the α1 sub-unit mediates the sedative effects of benzodiazepines (Rudolf et al. 1999; McKernan et al. 2000), while the α2 sub-unit mediates the anxiolytic effect of benzodiazepines (Low et al. 2000). The α5 sub-unit seemed to mediate the amnesic effects of benzodiazepines (Collinson et al. 2002). Interestingly, $GABA_A$ receptors containing the α4 sub-unit are insensitive to the anxiolytic effects of benzodiazepines (Wisden et al. 1991).

Most of these genetic studies, however, do not provide contiguous information about the specific brain circuits (e.g., amygdala, hippocampus) where

these $GABA_A$ receptor sub-units presumably produce their behavioral effects. Nevertheless, in vitro immunohistochemical and in situ hybridization data have provided important clues about the distribution of these sub-units in the brain, and in particular, in the hippocampus (e.g., Pirker et al. 2000; Seighart and Spert, 2002; Uusi-Oukari and Korpi 2010). These studies showed that the α1 and α2 sub-units are found throughout the brain (Uusi-Oukari and Korpi 2010), whereas the α5 sub-unit is found almost exclusively in the hippocampus (Sur et al. 1999). The hippocampus also contains high levels of α1, α2, and α4 sub-units (Uusi-Oukari and Korpi 2010). There is some evidence that the dorsal and ventral hippocampi contain different numbers of these sub-units. For example, the dorsal hippocampus contains more α1 and α4 receptor sub-units than the ventral hippocampus (Sotiriou et al. 2005), while the ventral hippocampus contains more α2 sub-units (Sotirou et al. 2005; Sarantis et al. 2008).

Although these data are important, they fall short of indicating the functional roles of the various $GABA_A$ receptor sub-units in the hippocampus. For example, it is tempting to speculate that the ventral hippocampus predominantly mediates the anxiolytic effects of benzodiazepines by virtue of its greater number of α2 sub-units. However, the dorsal hippocampus may still contain enough α2 sub-units to easily mediate the same effects. In addition, the genetic techniques that have suggested sub-unit functional specializations are not without methodological and interpretational problems (for reviews see Girlai, 1996; Stephens et al. 2002; Callaway, 2005; Geschwind and Konopka, 2009). In this light, it is important that specific compounds are now available which act as

agonists, antagonists, or inverse agonists at particular $GABA_A$ sub-units (e.g., TPA023, TB-21007; Atack, 2006; Chambers et al. 2003). Clearly, microinfusion of these sub-unit selective ligands into specific areas of the brain can be used to complement and extend genetic insights into $GABA_A$ sub-unit specializations. For example, if a sub-unit selective receptor ligand microinfused into the brain has behavioral effects consistent with those of a knock-out of the same sub-unit, this can provide powerful, neuroanatomically constrained, converging evidence for the role of that sub-unit in mediating the effects of benzodiazepines.

This general rationale forms the basis for the following set of experiments, which focus on the functional roles of $GABA_A$ receptor sub-units in the dorsal and ventral hippocampus. It is possible, for example, that some of the behavioral dissociations previously found after dorsal or ventral hippocampal lesions (e.g., Bannerman, 2004; McEown and Treit, 2010) may be related to specific sub-unit populations within these areas of the hippocampus.

With these considerations in mind, Experiment 1 will examine the behavioral effects of microinfusions of the selective α2 agonist TPA023 into the dorsal and ventral hippocampus. Ventral hippocampal TPA023 infusions should reduce anxiety-related behaviour by its agonist actions at α2 receptor sub-units, whereas it should have little effect on anxiety when infused into the dorsal hippocampus. If TPA023 does produce anxiolytic effects in specific areas of the hippocampus, this would provide spatially and temporally constrained corroboration of previous studies using α2 knock-out technologies.

The α5 $GABA_A$ sub-unit is thought to mediate the amnesic effect of benzodiazepines (Collinson et al. 2002), and the vast majority of α5 sub-units in the brain are located in the hippocampus, an area long implicated in memory processes (Sur et al. 1999). In Experiment 2, the role of α5 $GABA_A$ sub-units in the hippocampus on fear memory will be explored. Specifically, an *inverse* agonist of the α5 sub-unit (TB-21007) will be microinfused into the dorsal and ventral hippocampus, and its effects will be assessed on memory of a probe-shock experienced 24hrs earlier. TB-21007 has very low affinity for α1 receptor sub-units, where it also acts as an inverse agonist. At α2 sub-units, TB-21007 is an antagonist. According to these pharmacological data, TB-21007 infusions into the hippocampus should have little or no effect on unconditioned fear, or anxiety. However, as an inverse agonist at the α5 sub-unit, it may *facilitate* memory of a previous shock experience, 24hrs later. Whether this facilitation occurs in the dorsal or the ventral hippocampus will be particularly interesting.

In summary, the overarching purpose of the following experiments is to further explore the putative functions of $GABA_A$ sub-units in anxiety and memory, previously derived from genetic mutation of these sub-units, by microinfusing pharmacological ligands of these sub-units into specific regions of the hippocampus. Regardless of whether the results of these experiments are confirmatory or conflicting, they promise to add an important dimension to our knowledge of the role of hippocampal $GABA_A$ receptors in anxiety and fear memory.

Methods

Subjects

Eighty six Sprauge-Dawley rats (Ellerslie, Edmonton, Alberta, Canada) were used. Each animal weighed between approximately 150-250 grams upon arrival. Food and water were available ad libitum. Animals were individually housed in polycarbonate cages and kept on a 12:12 hour light/dark cycle (lights on at 0700 hours). Behavioral testing occurred during the light portion of the cycle.

Surgery

All surgeries conformed to the Society for Neuroscience Guidelines, CCAC guidelines, and to local animal care protocol # 6850312. Just prior to surgery, all subjects were randomly assigned to surgical conditions (dorsal or ventral hippocampal cannulae implants), injected with an analgesic (Rimadyl; 0.05cc, s.c.) to alleviate potential post-operative pain, and then injected with physiological saline to avoid dehydration (3cc, s.c., once before surgery). Subjects were anesthetised with Isoflurane gas (4% concentration in O_2 gas) and maintained at a 2% concentration throughout the duration of the surgery. Subjects were bilaterally implanted with 22-gauge, 8mm guide cannulae into the ventral hippocampus (-5.2 mm AP, -5.7 mm DV, +/- 5.6 mm lateral to midline) and 5mm guide cannulae into the dorsal hippocampus (-3.1 mm AP, - 3.3 mm DV, +/- 2.5 mm lateral to the midline). These anatomical coordinates were selected using the stereotaxic atlas of Paxinos and Watson, (1986). Two days after surgery, all external cannulae were tested for obstruction by re-inserting dummy cannulae into each cannulae tract, and hibitane (an antibacterial/antifungal cream) was re-applied to the surgical area.

Elevated plus-maze test

Upon arrival, rats were allowed three days to acclimatize to the colony room. After the acclimatization period all rats were individually handled for 5 minutes per day over a two day period. Surgeries were then performed with behavioural testing occurring six days post-surgery, during which time rats were handled daily during inspections for cannulae patency. The wooden plus-maze consisted of four elevated arms of which two (50 x 10 cm) were open and two were enclosed by walls (50 x 10 x 50 cm). During the 5 minute test period, the behavior of each rat in the elevated plus-maze was recorded on video tape for later analysis. Rats normally spend the majority of time in the enclosed arms, while avoiding the open arms. The number of entries rats made into the open arms, and the amount of time spent in the open arms, were quantified as 1) percentage of total entries (open entries/total entries times 100) and 2) percentage of total time (open time/total time times 100). These two measures of open-arm activity serve as indexes of anxiety in this test (Pellow and File, 1986). Increases in either of these indexes indicate anxiolysis (anxiety-reduction). The total number of entries into any arm and the total number of entries into the enclosed arms serve as measures of general activity in the plus-maze.

Shock-probe burying test

One day after the plus-maze test, each rat was handled for 5 minutes over the next two days. Rats were then habituated to the shock-probe test apparatus for 15 min over 4 successive days, without the shock-probe present. The apparatus consisted of a 40 × 30 × 40 cm Plexiglas chamber, with 5 cm of bedding material (wood

chips) spread evenly over the chamber floor. The Plexiglas shock-probe (6.5 cm long 0.5 cm in diameter) was helically wrapped with 2 copper wires and inserted through a hole in one of the walls of the chamber, 2 cm above the bedding material. The probe was electrified using a two-pole (bipolar AC) current reversal "square wave" output (Model H13-15, Colbourne Instruments) set at 2mA. Rats were then placed in the Plexiglas chamber, facing away from the electrified probe. The video-taped test began after rats' initial contact-induced probe-shock, and lasted 15 minutes. Rats typically receive one or two contact-induced shocks from the electrified probe during the test, which normally elicits "burying behavior" in which they spray bedding material toward or over the probe, with rapid, alternating movements of the forepaws, while avoiding further contact with the shock-probe. The number of contact-induced shocks, mean shock reactivity and still time (an inverse measure of general activity) were also measured. Mean shock reactivity was evaluated using a four point scale ranging from a slight flinch to jumping off the chamber floor (Pesold and Treit, 1992).

A reduction in the duration of probe-burying, in the absence of a decrease in general activity or shock-sensitivity is used as the primary index of anxiolysis in this test. A reduction in simple passive avoidance of the probe can also be used as a measure of anxiolysis in this test (Treit et al. 1986).

Infusion procedure

Rats were randomly assigned to vehicle or drug conditions. All microinfusions were bilateral. The vehicle control group received an intra-hippocampal infusion of a mixture of 10% DMSO and physiological saline (infusion rate: 1µl/1 min for

1 minute; pH 7.4) and the experimental group received an intra-hippocampal infusion of either TPA023 (Merck Sharpe and Dohme) or TB-21007 (Tocris) dissolved in the vehicle (concentration: 40µg/µl; infusion rate: 1µl/1min for 1 minute) resulting in an infusion of 40µg per hemisphere. Infusions were administered 10 minutes prior to the test session for all subjects. Infusions were administered with a 10µl Hamilton microsyringe using an infusion pump (Harvard Apparatus 22). The cannulae were left in place for 30 seconds after drug administration to allow for diffusion.

Elevated plus-maze

Rats were tested in groups representing six conditions 1) drug infusions into the dorsal hippocampus (i.e., TPA023 or TB-21007), 2) drug infusions into the ventral hippocampus (i.e., TPA023 or TB-21007) or 3) vehicle infusions into the dorsal or ventral hippocampus.

Shock-probe burying test I – acquisition phase

Rats were tested in the shock-probe apparatus under the same six conditions 1) drug infusions into the ventral hippocampus (i.e., TPA023 or TB-21007), 2) drug infusions into the dorsal hippocampus (i.e., TPA023 or TB-21007), or 3) vehicle infusions into the dorsal or ventral hippocampus.

Shock-probe burying test II - retention phase

Twenty-four hours after their first exposure to the electrified shock-probe, rats were re-tested in the same chamber, except 1) the probe was not electrified, and 2) microinfusions were not administered. The 15 min retention test was videotaped for later analysis. The amount of time rats spent in the half of the chamber farthest

away from the shock-probe was taken as a measure of fear memory (McEown and Treit, 2009).

Histology

After the completion of behavioural testing, rats were deeply anesthetised with Isoflurane gas (5% concentration in O_2 gas) and subsequently perfused with a 10% formalin solution. Their brains were removed and placed in specimen jars containing a 10% formalin solution. After forty-eight hours, brains were sectioned (60 µm), stained with thionin, and mounted on microscope slides. The locations of cannulae were confirmed using a light microscope. The behavioural data for rats with either one or both cannulae outside of the target area (dorsal or ventral hippocampus) were omitted from subsequent analysis.

Statistical analyses

All control measures (e.g., general activity) were assessed with ANOVA followed, where appropriate, with post-hoc, pair wise comparisons ($\alpha = 0.05$). All anxiety measures (percentage of open-arm activity, duration of burying) and the memory measure (duration of time on the side opposite the probe during the retention phase) were assessed using individual, a priori, pair-wise comparisons (t-tests, $\alpha = 0.05$, two-tailed). Burying behaviour underwent log transformations to correct for heterogeneity of variance.

Results

Experiment 1: α2 agonism using TPA023

Subjects and histology

Five rats did not make contact with the shock-probe during the acquisition session. Data from one rat was discarded from the analysis of plus-maze behaviour and one rat from the analyses of the shock-probe behaviour because their anxiety scores were more than three standard deviations from the mean. Data from an additional six rats was discarded due to misplaced cannulae. Four additional rats could not be tested due to blocked cannulae. This left 48 rats in the elevated plus-maze test [dorsal hippocampus TPA023 infusions = 17; ventral hippocampus TPA023 infusions = 15 and vehicle infusions = 16] and 41 rats in the shock-probe burying test [dorsal hippocampus TPA023 infusions = 16; ventral hippocampus TPA023 infusions = 13 and vehicle infusions = 12]. Figure 4-1 shows the correct bilateral placements in the dorsal and ventral hippocampus.

Elevated plus-maze

Between-group differences in the control measures were non-significant, indicating that TPA023 did not affect locomotor activity (number of closed arm entries ($F(2, 45) = .70$, p= .50); total number of arm entries ($F(2, 45) = .76$, p= .47; see Table 4-1 for descriptive statistics). Figure 4-2 shows the means and standard errors of the experimental and control groups' open-arm activity in the elevated plus-maze. Rats microinfused with TPA023 into the ventral hippocampus spent a significantly greater percentage of time in the open arms ($t(28) = 2.67$, $p < .01$) than controls (Figure 4-2) but rats infused with TPA023 into the dorsal hippocampus did not($t(30) = .26$, $p = .79$). The percentage of open-arm entries did not differ between rats infused into the ventral hippocampus ($t(29)$

= .73, p = .46) or dorsal hippocampus ($t(31) = -1.76$, p = .08) compared to controls (see Table 4-1 for descriptive statistics).

Shock-probe burying test I - acquisition phase

Still time ($F(2, 38) = .28$, p = .75) and number of shocks ($F(2, 38) = 1.08$, p = .34) were non-significant (see Table 4-1for descriptive statistics). However, a significant difference was observed between groups for shock reactivity ($F(2, 38) = 3.89$, $p < .05$). Tukey HSD post-hoc tests indicated that rats infused with TPA023 in the dorsal hippocampus were more sensitive to the probe-shock than rats infused with TPA023 in the ventral hippocampus ($p < .05$). Importantly, however, neither dorsal nor ventral TPA023 infused rats differed significantly from vehicle-infused controls in shock sensitivity ($p > .05$). TPA023 infusions into the ventral hippocampus significantly impaired burying behavior compared to saline infused controls ($t(22) = -2.07$, $p < .05$; see Fig. 4-3). Whereas, TPA023 infusions into the dorsal hippocampus did not significantly affect burying behaviour compared to saline infused controls ($t(26) = .71$, p= .47; see Fig. 4-4).

Shock-probe burying test II - retention phase

TPA023 infusions in the dorsal or ventral hippocampus had no effect on memory of the initial shock experience compared to vehicle controls: dorsal hippocampus ($t(26) = -1.34$, p = .18); ventral hippocampus ($t(23) = -1.22$, p = .23).

Experiment 2: α5 inverse agonism using TB-21007

Subjects and histology

Data from three rats was discarded due to misplaced cannulae. An additional two rats could not be tested due to blocked cannulae. One additional rat did not

receive a probe-shock and could not be tested. This left 28 rats in the elevated plus-maze [dorsal hippocampus TB-21007 infusions = 10; ventral hippocampus TB-21007 infusions = 9 and vehicle infusions = 9] and 25 rats in the shock-probe burying test [dorsal hippocampus TB-21007 infusions = 10; ventral hippocampus TB-21007 infusions = 7 and vehicle infusions = 8]. Figure 4-1 shows the correct bilateral placements in the dorsal and ventral hippocampus.

Elevated plus-maze

Between-group differences in the control measures were non-significant: number of closed arm entries; ($F(2, 25) = .53$, p= .59); total number of arm entries ($F(2, 25) = .39$, p= .67; see Table 4-1 for descriptive statistics). TB-21007 had no significant effect on either percent open-arm time or percent open-arm entries in either the ventral hippocampus (% open time: $t(16) = -.25$, p = .80; % open entries: $t(17) = .26$, p = .79) or the dorsal hippocampus (% open time: $t(17) = .26$, p =.79; % open entries: $t(17) = 1.71$, p = .10). The absence of an anxiolytic effect of TB-21007 is consistent with the fact it is an antagonist at the α2 sub-unit.

Shock-probe burying test I – acquisition phase

Still time ($F(2, 22) = .41$, p = .66), number of shocks ($F(2, 22) = 1.26$, p = .30) and shock reactivity ($F(2, 22) = .18$, p = .83) were all non-significant (see Table 4-1 for descriptive statistics).

Similar to the drug's null effects in the elevated plus-maze, TB-21007 did not significantly impair burying behavior compared to saline controls, whether it was infused into the dorsal or the ventral hippocampus (ventral hippocampus: $t(13) = -1.31$, p = .21; dorsal hippocampus: $t(16) = -1.37$, p = .18). These negative

data are also consistent with the fact that TB-21007 is an antagonist at α2 $GABA_A$ sub-units.

Shock-probe burying test II – retention phase

Unexpectedly, even though TB-21007 failed to produce an anxiolytic effect in the acquisition phase of the shock-probe burying test, 24hr later during the retention test it impaired memory of the initial shock-probe experience. Importantly, this memory impairment was specific to dorsal hippocampal infusions during acquisition ($t(16) = 2.05$, $p < .05$), since it did not occur after ventral hippocampal infusions during acquisition ($t(13) = .16$, $p = .87$, see Fig. 4-5).

Discussion

These results are the first to suggest that ventral hippocampal α2 $GABA_A$ receptor sub-units mediate anxiety. Specifically, an α2 agonist (TPA023) produced anxiolysis in two animal models of anxiety when infused into the ventral hippocampus but not when infused into the dorsal hippocampus. Our findings also suggest that dorsal hippocampal α5 $GABA_A$ sub-units are involved in fear memory. Infusions of an inverse α5 agonist (TB-21007) into the dorsal hippocampus during an acquisition trial impaired fear memory in a non-drugged retention test 24 hrs later; whereas, the same infusions into the ventral hippocampus did not affect fear memory during the retention test.

Muscimol is a non-sub-unit selective $GABA_A$ agonist. I previously found that infusing muscimol into the ventral hippocampus produced anxiolysis (see introduction). Our current results strongly suggest that the anxiolytic effects of muscimol in the ventral hippocampus were due to its agonist effects at the α2

$GABA_A$ sub-unit. TPA023, an agonist at the α2 sub-unit and antagonist at α1 and α5 sub-units, represented a test of this mechanism. The fact that this compound produced anxiolytic behavioral effects in the elevated plus-maze and shock-probe burying test when infused into the ventral hippocampus but not when infused into the dorsal hippocampus provides strong evidence that the anxiolytic effects of GABAergic agonists are mediated in part by α2 sub-units in the ventral hippocampus.

Genetic knock-out studies have also suggested that α2 $GABA_A$ sub-units mediate the anxiolytic effect of benzodiazepines (e.g., Low et al. 2000; Rudolf et al. 1999). However, these genetic "lesions" are associated with several methodological limitations. First, the knock-out may have unintended effects on neural development that can affect the behavioral phenotype during adulthood (e.g., Stephens et al. 2002; Tecott and Wehner, 2001). Second, the genetic background of the knock-out mice may affect whether or not the expected phenotype is expressed at all (e.g., Bucan and Abel, 2002; Phillips et al. 1999). Third, the expression of the knock-out phenotype can also vary significantly as a function of the specific laboratory environment in which it is tested, even when behavioral tests and environmental variables are strictly controlled (e.g., Crabbe et al. 1999). With these types of limitations in mind, our results using direct infusions of a specific α2 agonist provide powerful, converging evidence using two animal models of anxiety of the role $GABA_A$ α2 sub-units play in mediating anxiety. More importantly, these microinfusion studies suggest that not all α2 sub-units in the brain are involved in anxiety modulation, since I found that an α2 sub-

unit-specific ligand (TPA023) produced anxiolytic effects in the ventral but not the dorsal hippocampus.

On the other hand, the relationship of our findings to previous studies using α5 sub-unit knock-outs is more complex. In a previous study (McEown and Treit, 2010) I found that muscimol impaired memory of a prior shock-probe experience when infused into the dorsal hippocampus but not when infused into the ventral hippocampus, where it produced anxiolytic effects. Diazepam, also a non-specific $GABA_A$ sub-unit agonist, impaired passive avoidance in mice when infused directly into the dorsal hippocampus, also indicating impaired fear memory (Kim et al. 2011). NS11394, a $GABA_A$ sub-unit agonist with higher efficacy at the α5 compared to α3, α2, and α1 sub-units, also impaired passive avoidance when administered peripherally (Mirza et al. 2008). Genetic knock-out of the α5 sub-unit, in contrast to the effects of $GABA_A$ agonists, produced memory facilitation in the Morris water maze, a test of rats' spatial memory (Collinson et al. 2002). Genetic knock-out of the α5 sub-unit also facilitated fear memory in a trace fear conditioning paradigm (Crestani et al. 2002).This facilitatory effect of α5 sub-unit knock-outs on fear memory is consistent with the muscimol, diazepam and NS11394 data above, which suggested that *agonism* of this sub-unit impairs fear memory.

Since *inverse* agonists typically produce effects opposite to that of full agonists I also expected that an α5 inverse agonist should facilitate memory. Indeed, peripheral administration of a variety of α5 inverse agonists (L-655708, R04938581, TB-21007 or α5IA) are known to facilitate spatial memory (Atack et

al. 2006; Ballard et al. 2009; Chambers et al. 2003; Dawson et al. 2006). In stark contrast to these findings, however, I found that intra-hippocampal infusion of a high affinity α5 inverse agonist (TB-21007) actually *impaired* memory of a shock-probe experience when it was infused into the dorsal hippocampus. This impairment was probably not due to sedative or anxiolytic effects since TB-21007 is an inverse agonist at the α1 sub-unit and an antagonist at the α2 and α3 sub-units.

Thus, the effects I found with TB-21007 are contrary to expectations based on the previous literature. However, these apparent contradictions may be specific to the memory task that is given animals. It is quite possible that the role of the α5 sub-unit in fear memory is different than its role in spatial memory, where most of the memory facilitation effects of α5 inverse agonists have been demonstrated (e.g., Atack et al. 2006; Ballard et al. 2009; Chambers et al. 2003; Dawson et al. 2006).

It is also possible that the fear memory impairments seen after full agonists such as diazepam or muscimol were infused into the dorsal hippocampus resulted not from their actions at the α5 sub-unit, but from their actions at the α1 sub-unit. Indeed, genetic knock-out of the α1 sub-unit *blocks* the memory impairment typically seen after diazepam administration (Smith et al. 2012). However, genetic knock-out of the α1 sub-unit also blocks sedation typically seen after diazepam administration (Rudolf et al. 1999). Based on these data, the results of Smith et al. could have resulted from the absence of benzodiazepine-induced sedation rather than to a reversal of diazepam-induced memory

impairment. In either case, however, 'memory effects' attributed to the α5 sub-unit could be due, either partially or completely, to effects at the α1 sub-unit.

Nevertheless, and consistent with the current results, microinfusion of the inverse α5 agonist RY024 into the dorsal hippocampus impaired conditioned fear in rats (Bailey et al. 2002). RY024 has a lower binding affinity at the α1 sub-unit (Ki = 26.9nM) compared to its affinity for the α5 sub-unit (Ki = 1.6nM see Liu et al. 1996; Chambers et al. 2003). Furthermore, TB-21007 is an *inverse agonist* at α1 receptor sub-units and would be expected to antagonize any sedative effect mediated by this sub-unit (i.e., Smith et al. 2012). Thus, the impairment in conditioned fear observed by Bailey et al. and in the present experiment seems to be due to inverse agonism at the α5 receptor sub-unit.

On the other hand, recent evidence suggests that α5 sub-units may mediate sedation, rather than memory. Several compounds with low α1 sub-unit agonist efficacy and moderate to high agonist efficacy at α5 sub-units were assessed in the Morris Water maze (Savic, 2010). These compounds produced sedation, but no spatial memory impairments. If α5 sub-units in the hippocampus mediate sedation I would expect that inverse agonists of the α5 sub-units would decrease sedation. In the present experiments, however, TB-21007, an inverse agonist of α5 sub-units produced no effect on general activity in either the elevated plus-maze (i.e., total number of arm entries) or shock-probe burying test (i.e., still time). More importantly, TB-21007 infusions occurred a day prior to retention testing; therefore, it is unlikely TB-21007 influenced sedation during the retention test period in which memory of the shock-probe experience was assessed.

Inverse agonists typically produce effects opposite to agonists. However, high doses of certain $GABA_A$ agonists, for example the benzodiazepines lorazepam and midazolam, can produce the opposite behavioural effect (i.e., anxiogenesis; McKenzie and Rosenberg, 2010) to what is usually observed at low to moderate doses (i.e., anxiolysis) (Bond 1998). Thus, the high dose of TB-21007 used in our experiment (40µg/µl) may have resulted in an agonist effect at hippocampal α5 sub-units, thereby impairing memory. Intra-hippocampal dose-effect studies of TB-21007 are needed to test this hypothesis.

In summary, this study was the first to examine the separate roles of α2 sub-units in the ventral hippocampus and α5 sub-units in the dorsal hippocampus in mediating anxiety and fear memory. I demonstrate for the first time that ventral hippocampal $GABA_A$ α2 sub-units mediate anxiety, and I provide evidence that infusing an inverse α5 agonist into the dorsal hippocampus impairs fear memory. These results provide important neuroanatomical insights into the complex roles that $GABA_A$ receptor sub-units perform in anxiety and fear memory.

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TPA023				TB-21007		
	Dorsal hippocampus	Ventral hippocampus	Controls	Dorsal hippocampus	Ventral hippocampus	Controls
Plus-maze	*(n = 17)*	*(n = 15)*	*(n = 16)*	*(n = 10)*	*(n = 9)*	*(n = 9)*
Closed arm entries	7.65 (.75)	6.93 (.94)	6.25 (.85)	5.80 (.68)	5.22 (.96)	6.67 (1.25)
Total arm entries	10.76 (1.24)	13.07 (1.92)	10.50 (1.60)	10.20 (.78)	9.00 (1.15)	9.11 (1.27)
% Open arm entries	27.85 (4.23)	43.35 (4.54)	38.67 (4.42)	42.76 (5.43)	43.46 (6.50)	23.30 (8.50)
Shock-probe	*(n = 16)*	*(n = 13)*	*(n = 12)*	*(n = 10)*	*(n = 7)*	*(n = 8)*
Still time	65.94 (33.01)	98.15 (55.01)	56.75 (22.45)	110.40 (40.53)	224.43 (97.52)	114.25 (64.76)
Shock number	1.94 (.24)	1.62 (.21)	2.17 (.29)	2.30 (.26)	2.29 (.52)	3.00 (.33)
Shock reactivity	1.86 (.13)	1.37 (.12)	1.55 (.10)	1.75 (.14)	1.88 (.18)	1.77 (.18)

Table 4-1: Mean ($\pm$ S.E.M.): 1) closed arm entries, 2) total arm entries, 3) percentage of open arm entries, 4) still time, 5) shock number, 6) shock reactivity.

Figure 4-1: Illustrations of coronal sections of the rat brain adapted from Paxinos and Watson (1986) displaying the approximate location of dorsal and ventral hippocampal infusion sites in Experiments 1 and 2. The numbers indicate A–P coordinates relative to bregma.

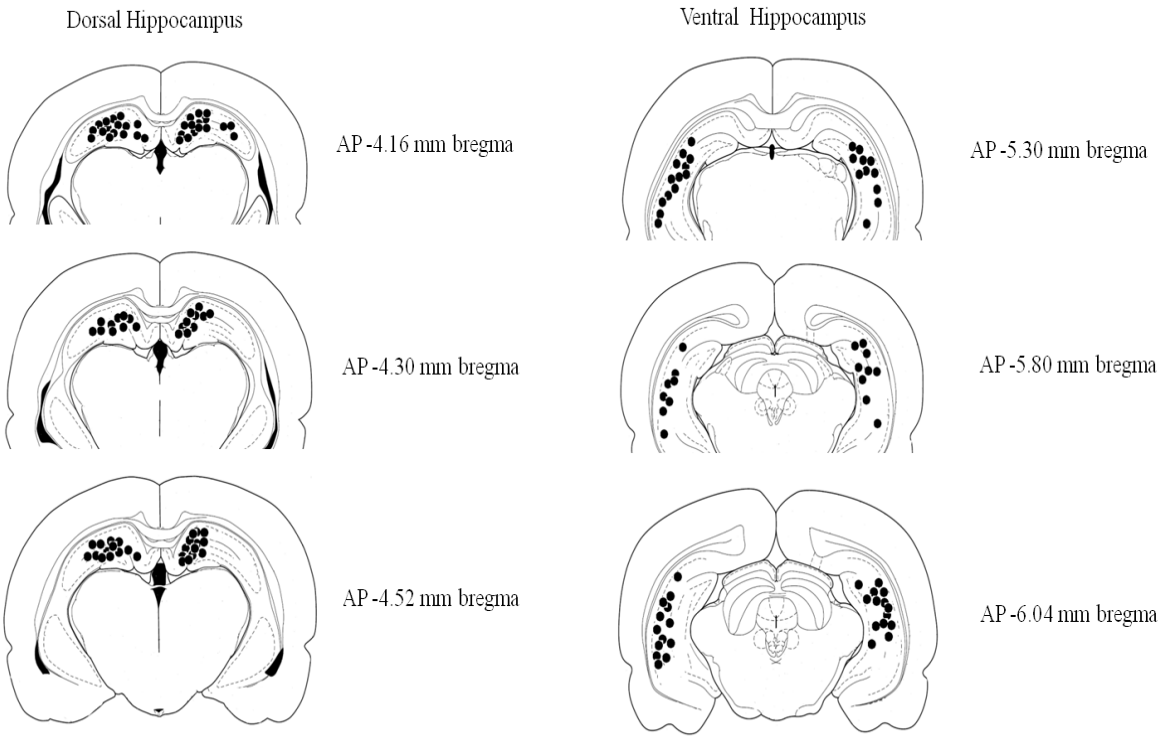

Dorsal Hippocampus
Ventral Hippocampus
AP -4.16 mm bregma
AP -5.30 mm bregma
AP -4.30 mm bregma
AP -5.80 mm bregma
AP -4.52 mm bregma
AP -6.04 mm bregma

Figure 4-2: Mean (± S.E.M.) percentage of open arm time (experiment one) of dorsal hippocampus (TPA023), ventral hippocampus (TPA023) and control (vehicle) rats.

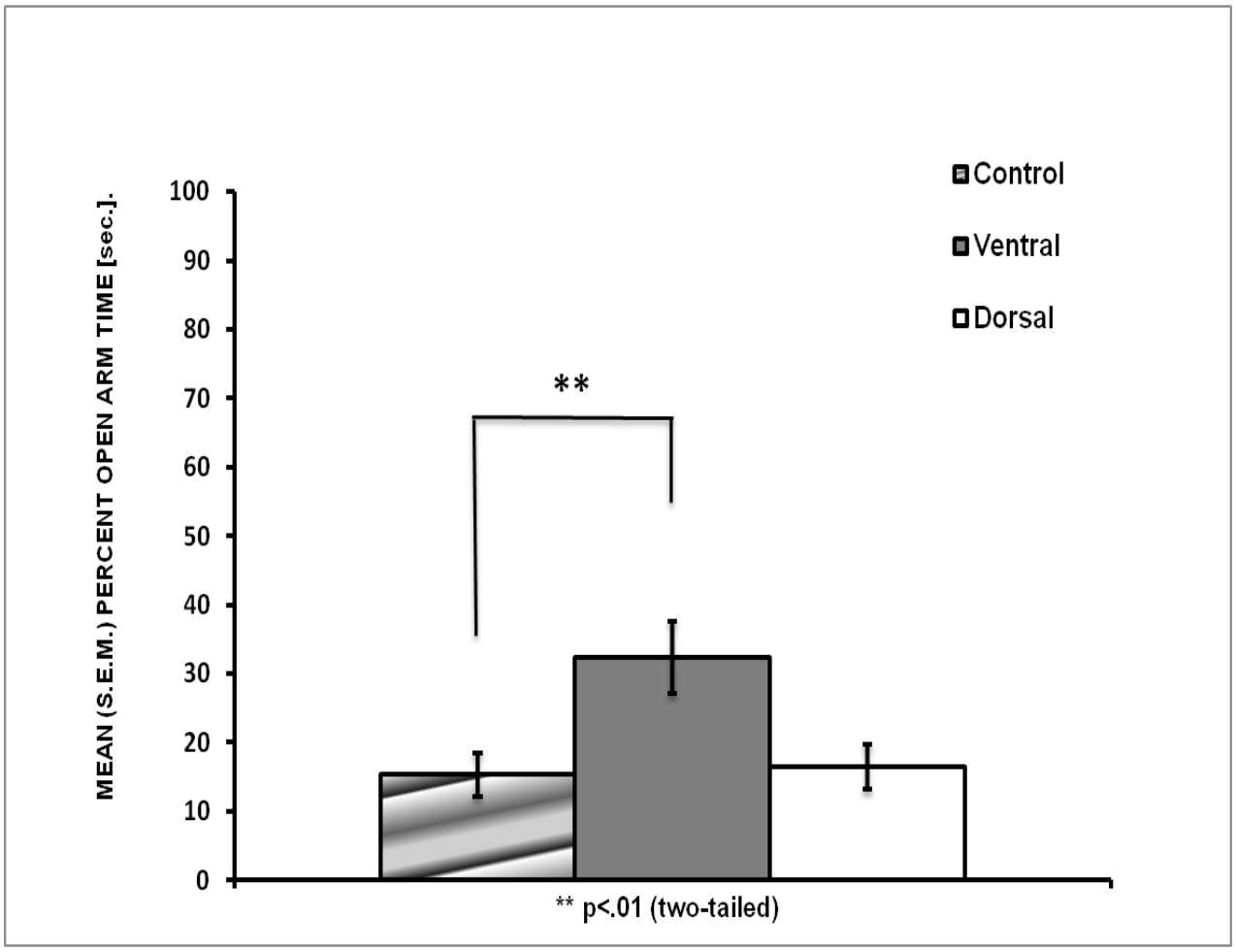

MEAN (S.E.M.) PERCENT OPEN ARM TIME [sec.].
100
90
80
70
60
50
40
30
20
10
0
**
Control
Ventral
Dorsal
** p<.01 (two-tailed)

Figure 4-3: Mean (± S.E.M.) duration of burying time (experiment one) of ventral hippocampus (TPA023) and control (vehicle) rats.

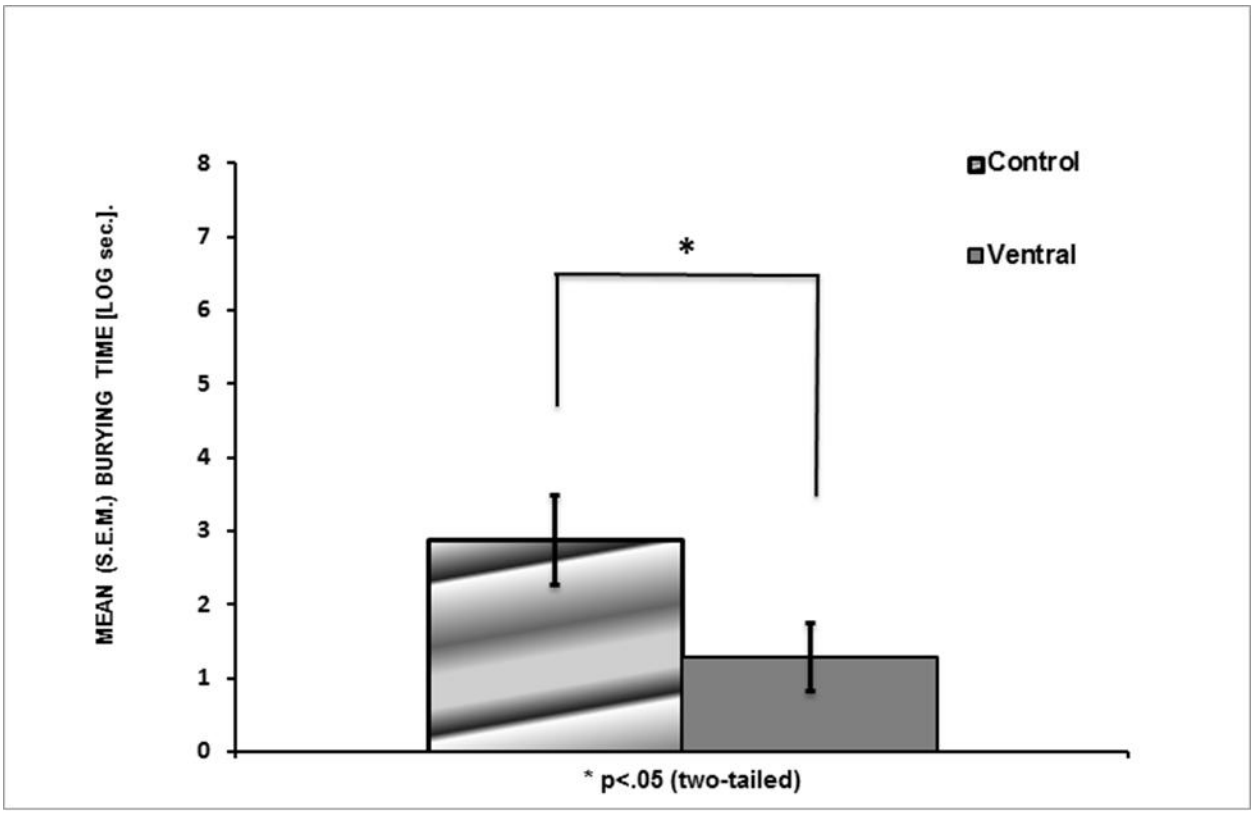

MEAN (S.E.M.) BURYING TIME [LOG sec.].
8
7
6
5
4
3
2
1
0
*
Control
Ventral
* p<.05 (two-tailed)

Figure 4-4: Mean (± S.E.M.) duration of burying time (experiment one) of dorsal hippocampus (TPA023) and control (vehicle) rats.

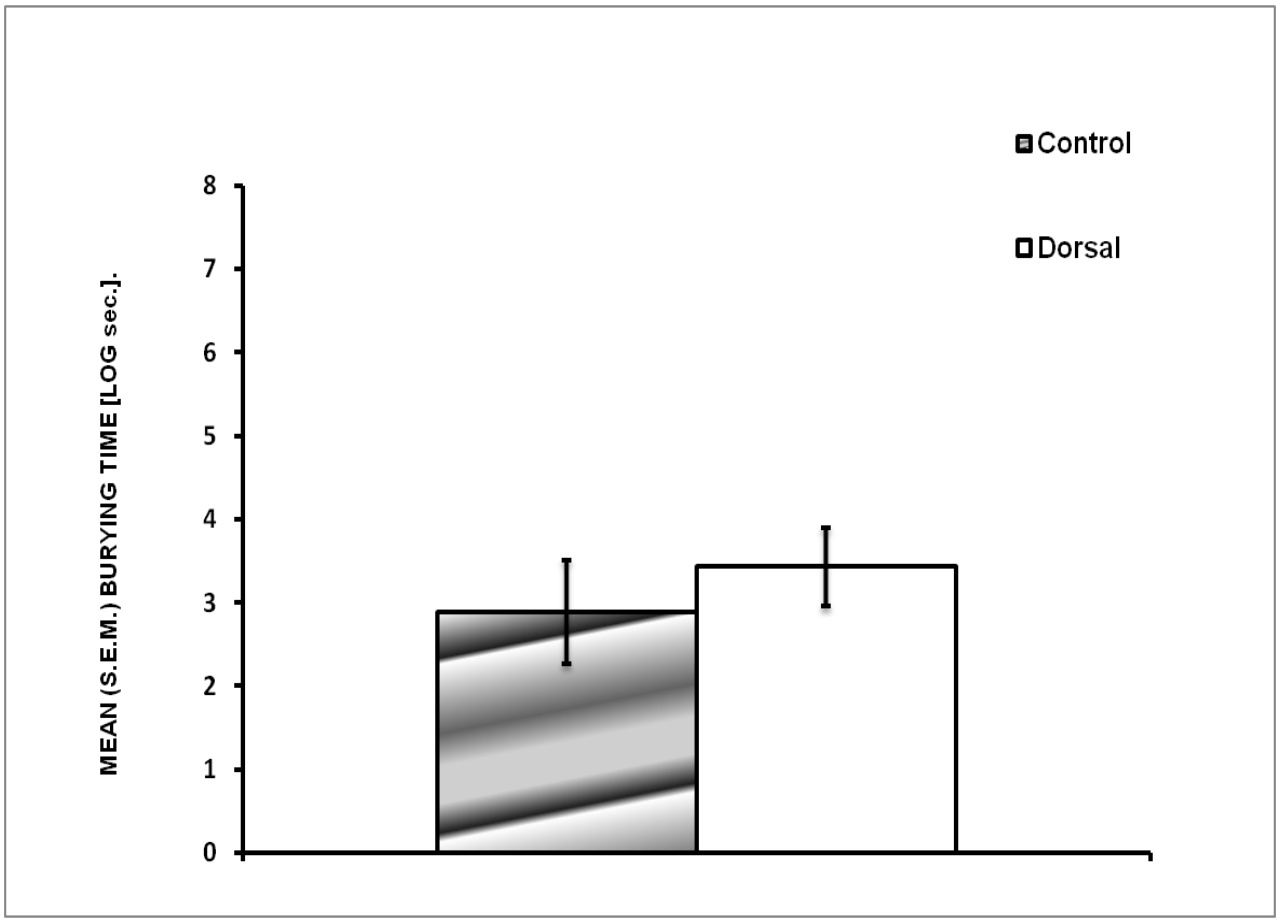
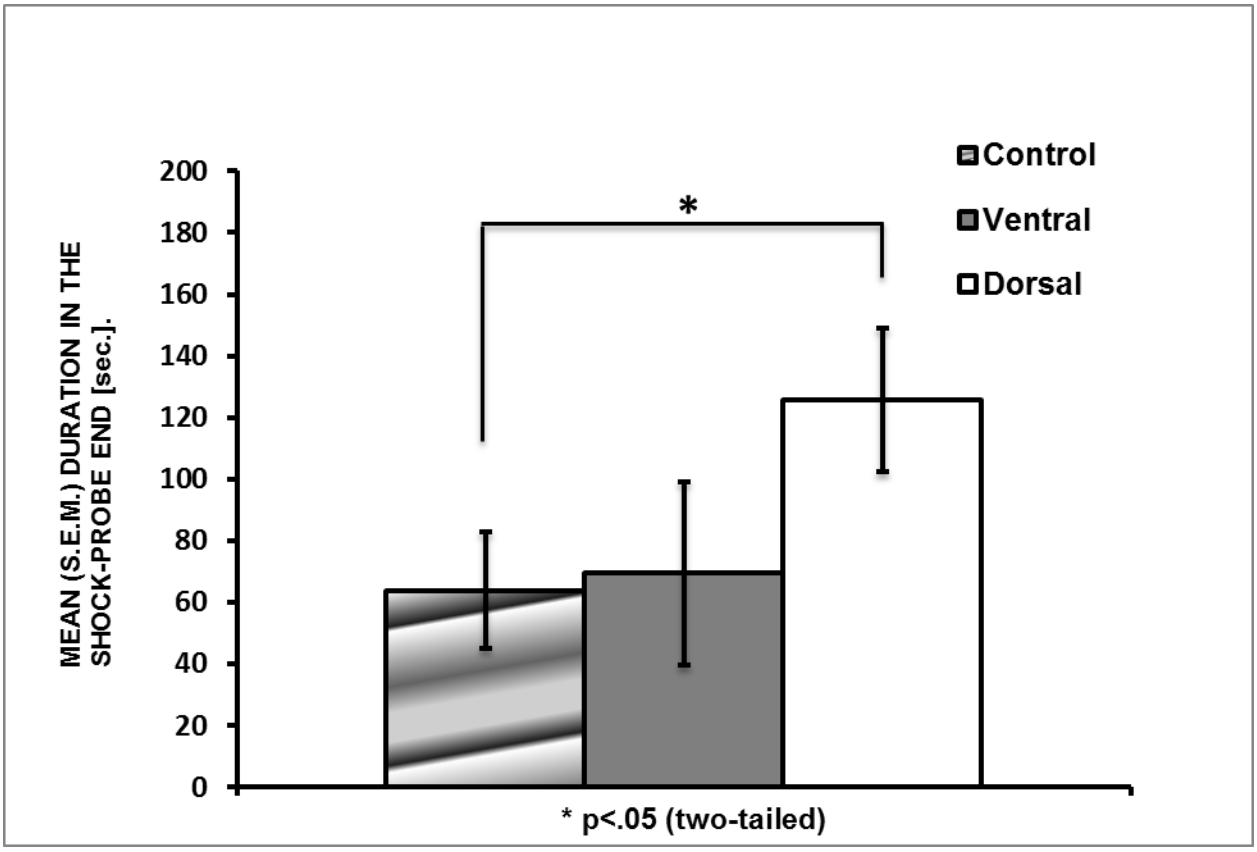

Control
Dorsal
MEAN (S.E.M.) BURYING TIME [LOG sec.].
8
7
6
5
4
3
2
1
0

Figure 4-5: Mean (± S.E.M.) duration of time spent in the shock-probe end of the chamber (experiment two) of dorsal hippocampus (TB-21007), ventral hippocampus (TB-21007) and control (vehicle) rats.


MEAN (S.E.M.) DURATION IN THE SHOCK-PROBE END [sec.].
200
180
160
140
120
100
80
60
40
20
0
*
Control
Ventral
Dorsal
* p<.05 (two-tailed)

Chapter 5

General Discussion

This dissertation provides evidence suggesting that mineralocorticoid and $GABA_A$ receptors in the ventral hippocampus mediate anxiety. Antagonism of mineralocorticoid receptors in the ventral hippocampus reduced anxiety in the shock-probe burying test and the elevated plus-maze, as did agonism of $GABA_A$ receptors in the ventral hippocampus. More importantly, specifically stimulating $GABA_A$ α2 receptor sub-units in the ventral hippocampus produced behavioral effects comparable to those of intra-hippocampal infusion of a standard, benzodiazepine anxiolytic drug, diazepam, a non-specific agonist at the benzodiazepine/$GABA_A$ receptor site. These results are consistent with the view that the α2 sub-unit of the $GABA_A$ receptor mediates the anxiolytic effects of benzodiazepines.

On the other hand, the role in anxiety of mineralocorticoid and $GABA_A$ receptors in the *dorsal* hippocampus is still not entirely clear. A mineralocorticoid receptor antagonist infused into the dorsal hippocampus produced an anxiolytic effect in the shock-probe test but not in the elevated plus-maze test. Diazepam infused into the dorsal hippocampus did have an anxiolytic effect in the elevated plus-maze, but a selective, $GABA_A$ α2 receptor sub-unit agonist infused into the dorsal hippocampus failed to affect anxiety, either in the elevated plus-maze, or in the shock-probe burying test. Unlike the reliable anxiolytic effects of these compounds in the ventral hippocampus, their anxiolytic effects in the dorsal hippocampus were inconsistent, and often limited to a single behavioral index of anxiety.

By introducing a twenty-four hour delay between an initial shock-probe event ("acquisition") and a subsequent test in the same apparatus but with a non-electrified probe ("retention"), I was able to distinguish drug effects on unconditioned fear and anxiety in the first instance, and drug effects on conditioned fear (or fear memory) in the second instance. As expected from previous results, intra-dorsal hippocampal infusion of a $GABA_A$ α2 receptor sub-unit agonist did not affect anxiety in the acquisition phase, or fear memory in the retention phase. On the other hand, the $GABA_A$ α5 receptor sub-unit has been shown to be involved in memory function in previous experiments (e.g., Collinson et al. 2002). In the current experiments, I showed that intra-dorsal hippocampal infusion of an inverse agonist of $GABA_A$ α5 sub-units impaired fear memory (i.e., a "retention" deficit; see above).

Another possibility is that $GABA_A$ α5 sub-units mediate anxiety, not memory. Bailey et al. (2002) found that infusing a $GABA_A$ α5 inverse agonist into the dorsal hippocampus impaired fear memory and increased anxiety (i.e., freezing behaviour). Therefore, it is possible that rats who received dorsal hippocampal TB 21007 infusions prior to acquisition were more anxious than controls in the shock-probe burying test during acquisition (i.e., during the initial learning phase). When TB 21007 infused rats were tested for fear memory 24hrs later during retention they may have been in a less anxious state compared to acquisition. Therefore, potential differences in rats' anxiety between acquisition and retention may have been interpreted as an effect on 'fear memory.' However, agonism of α2 sub-units in the ventral hippocampus reduced anxiety during

acquisition, yet these rats did not display any differences in fear memory during retention compared to controls. In addition, if dorsal hippocampal α5 inverse agonism produced anxiogenesis during acquisition I would expect increased burying behaviour in these rats during acquisition compared to controls. However, rats that received dorsal hippocampal TB 21007 infusions did not display differences in burying behaviour during acquisition compared to controls. Therefore, potential differences in rats' anxiety during acquisition and 24hrs later during retention are not likely explanations for probe avoidance impairments observed during retention.

Taken together, the results described in this dissertation suggest that hippocampal mineralocorticoid receptors and hippocampal benzodiazepine/$GABA_A$ receptors have opposite effects on anxiety. Specifically, stimulating benzodiazepine/$GABA_A$ receptors in the hippocampus generally decreased anxiety, while inhibiting mineralocorticoid receptors decreased anxiety. These findings are consistent with past research suggesting that cortisol (corticosterone in rodents) and GABA at $GABA_A$ receptors play opposite roles in regulating organisms' responses to environmental stressors (Mikkelsen et al. 2005; Takamatsu et al. 2003; Zhao et al. 2012). Furthermore, cortisol is higher in human patients with anxiety disorders compared to healthy individuals (e.g., Abelson and Curtis 1996; Maes et al. 1998; Nesse et al. 1984) and benzodiazepine $GABA_A$ agonists attenuate this increase (e.g., Curtis et al. 1997; Pomara et al. 2005).

To reiterate, the $GABA_A$ α2 sub-unit agonist TPA023 and the non-sub-unit selective drug diazepam each reduced anxiety when these compounds were separately infused into the ventral hippocampus, but only diazepam had anxiolytic effects in the dorsal hippocampus. These conflicting results might be explained by differences in *efficacy* between these compounds at $GABA_A$ α2 receptor sub-units in the dorsal hippocampus. That is, TPA023 is less effective than diazepam at modulating chloride conduction through $GABA_A$ receptor channels, which itself produces neuronal inhibition (Atack et al. 2006; Dawson et al. 2006). Therefore, it seems possible that diazepam was more effective than TPA023 at activating α2 sub-units in the dorsal hippocampus and thus more effective at reducing anxiety. To further add to this point, the dorsal hippocampus contains significantly fewer $GABA_A$ α2 receptor sub-units compared to the ventral hippocampus (Sotiriou et al. 2005).

Another possibility is that the reduction in anxiety observed after infusing diazepam into the dorsal hippocampus was mediated by $GABA_A$ α1 receptor sub-units. For example, knock-out mice that lack α1 sub-units display anxiogenesis in animal models of anxiety, which implies that the α1 sub-unit in part mediates anxiety (Ye et al. 2010). Additionally, rats peripherally administered the $GABA_A$ α1 agonist DOV51892 or the $GABA_A$ α1 agonist ocinaplon were significantly less anxious in the Vogel conflict test and elevated plus-maze compared to controls (Lippa et al. 2005; Popik et al. 2006). Importantly, doses of DOV51892 or ocinaplon that were effective at reducing anxiety had no effect on sedation, which itself is an effect previously associated with the α1 sub-unit (e.g., McKernan et al.

2000; Lippa et al. 2005; Popik et al. 2006). It should also be noted that the dorsal hippocampus contains a greater number of $GABA_A$ α1 receptor sub-units compared to the ventral hippocampus (Sotiriou et al. 2005). Taking these findings into consideration, the reduction in anxiety I observed after infusing the non-selective $GABA_A$ α1 receptor agonist diazepam into the dorsal hippocampus may have been mediated by $GABA_A$ α1 sub-units in the dorsal hippocampus. Furthermore, the null effect on anxiety that was observed after infusing the $GABA_A$ α2 sub-unit agonist TPA023 into the dorsal hippocampus may have occurred because TPA023 has no effect at $GABA_A$ α1 sub-units. Future studies are needed to test these conjectures.

Other research suggests that hippocampal $GABA_A$ receptor function is regulated in part by CORT levels in the brain. The acute effect of adrenalectomy is to *increase* the number of hippocampal benzodiazepine receptor binding sites in mice (Miller et al. 1988) and *increase* $GABA_A$ α2 receptor sub-unit mRNA in the hippocampus (Orchinik et al. 1994). Conversely, when CORT levels are chronically increased in rats by surgically implanting CORT pellets subcutaneously, the amount of $GABA_A$ α2 receptor sub-unit mRNA was *reduced* in the hippocampus (Orchinik et al. 1995) and the sensitivity of $GABA_A$ receptors to activation by GABA was also reduced in the hippocampus (Orchinik et al. 2001). Furthermore, chronic stress exposure is associated with reduced neuronal inhibition (i.e., impaired chloride ion channel function at $GABA_A$ receptors) produced by activating $GABA_A$ receptors (Drugan et al. 1989). Finally, it is important to note that facilitating hippocampal $GABA_A$ receptor-mediated

neuronal inhibition prevents cell death in the hippocampus (Johansen and Diemer 1991). Taken together these results suggest that chronically increasing CORT in the brain decreases the number of $GABA_A$ receptors in the hippocampus and reduces their ability to produce neuronal inhibition. Furthermore, this reduction in neuronal inhibition may then lead to cell death in the hippocampus and subsequent impairments in behaviour associated with hippocampal damage (e.g., depression and anxiety are produced) (e.g., Brown et al. 2004; Kajiyama et al. 2010).

Finally, the amygdala and hippocampus are thought to exert opposite roles in regulating the HPA system through excitatory and inhibitory connections with the hypothalamus. Hippocampal glutamatergic neurons project to the hypothalamus, inhibiting the release of CRH by activating GABAergic neurons in the hypothalamus (Herman et al. 2005). A reduction in CRH release results in less cortisol release from the adrenal cortex via the HPA system (Herman et al. 2005). In this way, the hippocampus could serve as a negative feedback mechanism within the HPA system. On the other hand, GABAergic neurons in the amygdala project to the hypothalamus and inhibit post-synaptic GABA neurons thereby reducing neuronal inhibition in the hypothalamus (Herman et al. 2005). The end result of activating these GABAergic amygdala projections increases CRH release (Herman et al. 2005). This increase in CRH *potentiates* cortisol release from the adrenal cortex via the HPA system. Thus, the amygdala could play an opposite role in regulating the HPA system by serving as a positive feedback mechanism.

In conclusion, glutaminergic and GABAergic neurotransmitter systems regulate the HPA system via the hippocampus and amygdala.

This dissertation provides evidence suggesting that mineralocorticoid and $GABA_A$ receptors in the dorsal and ventral hippocampus mediate anxiety. Furthermore, this research suggests that $GABA_A$ α2 sub-units in the ventral hippocampus and $GABA_A$ α5 sub-units in the dorsal hippocampus separately mediate anxiety and fear memory. These findings provided important neuropharmacological insights into the neural mechanisms of anxiety and fear memory.

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